



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p><b>(54) Title:</b> PROCESS FOR INHIBITING THE GROWTH OF A CULTURE OF LACTIC ACID BACTERIA, AND OPTIONALLY          LYSING THE BACTERIAL CELLS, AND USES OF THE RESULTING LYSED CULTURE</p>		
<p><b>(57) Abstract</b></p> <p>The invention provides a process for inhibiting the growth of a culture of lactic acid bacteria, or a product containing such culture e.g. a cheese product, in which in the cells of the lactic acid bacteria a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria is produced <i>in situ</i>, the gene encoding said holin being under control of a first regulatable promoter, said holin being capable of exerting a bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired. It is preferable that additionally a lysin obtainable from lactic acid bacteria or their bacteriophages is produced <i>in situ</i> in the cells of the lactic acid bacteria, the gene encoding said lysin being under control of a second regulatable promoter, whereby the produced lysin effects lysis of the cells of the lactic acid bacteria. The second regulatable promoter can be the same as the first regulatable promoter and the genes encoding the holin and the lysin, respectively can be placed under the same regulatable promoter in one operon. Preferably the promoters are regulatable by the food-grade ingredients or parameters. Other uses of the invention include preparing a mixture of peptides which are modified by peptidases freed after the lysis, using the lysed culture as a bactericidal agent against spoiling bacteria or pathogenic bacteria for improving the shelf life of a product containing the lysed culture.</p>		

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Process for inhibiting the growth of a culture of lactic acid bacteria, and optionally lysing the bacterial cells, and uses of the resulting lysed culture

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Background of the invention and prior art

The invention relates to a process for inhibiting the growth of a culture of lactic acid bacteria, and optionally lysing the cells of said bacteria.

In this specification the following abbreviations of names of micro-organisms are used:

- E.* = *Escherichia*, e.g. *E. coli*,
- L.* = *Lactococcus*, e.g. *L. lactis*,
- 15 *M.* = *Micrococcus*, e.g. *M. lysodeikticus*,
- S.* = *Streptococcus*, e.g. *S. faecalis* and *S. pneumonia*.

In one aspect the invention relates to a process for the lysis of a culture of lactic acid bacteria, or a product containing such culture, by means of a lysin e.g. in producing a fermented food product, e.g. in cheese-making. Such a process is known from WO 90/00599 (AGRICULTURAL & FOOD RESEARCH COUNCIL (AFRC), M.J. Gasson, published 25 January 1990, ref. 1). According to that patent specification the lysin from a *Lactococcus* (preferably prolate-headed) bacteriophage was used to lyse bacterial starter cultures during cheese-making. Exemplified was the lysin of the bacteriophage  $\phi$ ML3 of *Lactococcus lactis* ML3. In particular, the lysin can be added to a cheese product or a cheese precursor mixture, e.g. after whey removal, milling and salting. However, this solution has the disadvantage that thorough mixing of the contents of the lysed cells with the cheese product is not easily obtained. Another disadvantage is that the lysin was produced by *Escherichia coli* cells, which are not food-grade. It is explicitly stated if the cell wall of the host cell is not itself degraded by the lysin then the lysin secreting transformed host may be useful in suppressing populations of bacteria which are susceptible to lysis by the lysin. Nothing is mentioned regarding addition of a transformed host cell in improving cheese flavor, certainly not a transformed lactic acid bacterium.

As an alternative it is suggested in that patent specification "to encapsulate the lysin so that the timing of its addition is not important. The encapsulating agent dissolves after the cheese-making process is complete

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thus not affecting the starter bacteria before their role in acidification was complete."

This suggested alternative has the disadvantages, that (a) an encapsulating material has to be used, and (b) said material must not dissolve before the end of the cheese making process. Moreover, if the encapsulated lysin is added at the beginning of the cheese-making process, e.g. while adding the cheese starter culture to the milk, about 90% of it is removed with the whey. Thus one has to add about tenfold the required effective amount, which is economically not attractive. In a later publication C.A. Shearman, K. Jury & M.J. Gasson (Feb. 1992, ref. 2) described an autolytic *Lactococcus lactis* expressing a cloned lactococcal bacteriophage  $\phi$ vML3 lysin gene. In particular they stated that

"(e)xpression of the cloned lysin did not impair the ability of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains to metabolize lactose, to clot milk and produce acid (data not shown)".

It was suggested that during the exponential phase the lysin would not, or would insufficiently be expressed. It would only be expressed in sufficient amounts to lyse an appreciable proportion of the cells during the stationary phase, which occurs at the end of the normal fermentation process. The article illustrates that maintenance of transformed lactococcal strains could be a problem. Maintenance at a temperature below 30°C slightly delayed the onset of lysis but at 30°C regrowth of lysin resistant bacteria occurred. As alternative buffering in a sucrose medium with a sucrose percentage higher than 20% was given. This does not seem to be suitable in a process of fermentation like cheese making where the fermentation step occurs at 30°C or higher and the presence of more than 20% sucrose is not acceptable.

Furthermore, at the end of that publication it was indicated that expression in the stationary phase is not completely controlled. In addition the use of osmotic buffer in a cheese maturing process is probably not very efficient timewise when taking into consideration the length of time required for a Gouda cheese immersed in a brine bath to achieve the desired degree of salt flavour the osmotic effect of salt concentration is not going to be very quick. The cheddar cheese making process would probably be more suitable as the salt addition step is more efficient, however, still requires a mixing step.

Both disclosures described the use of a lysin originating from a lactococcal bacteriophage lysin, that means an enzyme produced in nature by an undesired substance like a bacteriophage, because bacteriophage contaminations are a major problem in large scale industrial dairy fermentation processes.

In a review article R. Young (1992, ref. 4) gives a survey of the state of the art on bacteriophage lysis, both mechanism and regulation. Especially in the section "Lysis in Phage Infections of Gram-Positive Hosts" on pages 468-472 it was indicated that the DNA sequence found by Shearman c.s. (1989, ref. 5), which DNA sequence seems to be the same as that given in ref. 1, is probably not correct and that the deduced amino acid sequence might be quite different due to a mutation causing a phase shift in the reading frame. More particularly it is speculated that the DNA sequence of the lysin gene like the pneumococcal phage associated lysin genes had no signal sequence which could account for secretion across the cytoplasmic membrane, however, this was puzzling in view of the absence of a typical N-terminal signal sequence which raises the question of how the lytic enzymes escape the cytoplasm and gain access to the cell wall.

In the above mentioned review of R. Young (1992, ref. 4, especially on page 469 in the paragraph bridging both columns and pages 472-473 in the section HOLIN FAMILY) it is argued that an additional protein is required for the action of bacteriophage lysins on the cell wall of infected cells. This additional protein is required for the access of the murein hydrolase, which is the more scientific name for the bacteriophage lysin, to its murein substrate. In that review the term "holin" was used for this additional protein. It was described in that review that the holin makes perforations in the cell wall enabling the lysins to pass the membrane so that subsequently the lysins can hydrolase the murein part of the cell wall. In this specification "holin" also means a protein or peptide required for the access of a lysin to its substrate, the murein part of the cell wall.

In the Young et al review it is stated that the realignment of the Shearman sequence and assumptions of a sequencing error obscuring a start codon does present a possible basis for establishing the requirement for a holin to effect the release of this phage encoded murein hydrolase. Young et al. further state "If our analysis so far has taught us anything it is that any phage with a lysozyme gene should have a holin gene". This statement is however contradicted somewhat further on

in the same article where a lys A clone of mvl which does not appear to possess a holin encoding sequence is illustrated as exhibiting lytic activity.

5 Young et al further examined the putative holin family and disclose 8 different proteins unrelated in primary sequence for which genetic or physiological evidence of holin function exists. Some postulations concerning structure and function are made, however nothing definite appears to be settled regarding this issue. They indicate that as these proteins are small, hydrophobic, without enzyme function and 10 lethal this is an array of characteristics not likely to attract legions of biochemists. This field is thus illustrated as being quite complex with little factual knowledge and a deal of speculation.

In a publication of Ward c.s. (1993; ref. 6) it is also suggested that the sequence of Shearman et al. (1989; ref. 5) is probably 15 not correct. Comparison with a very similar phage lysin gene confirmed that a frame shift in the Shearman et al. (ref. 5) sequence is needed for aligning the two DNA sequences. Moreover, this comparison teaches that the real phage lysin is encoded by an ORF that is probably 45 bases longer than disclosed by Shearman et al. (ref. 5).

20 C. Platteeuw and W.M. de Vos (1992, ref. 3) described the location, characterization and expression in *Escherichia coli* of lytic enzyme-encoding gene, *lytA*, of *Lactococcus lactis* bacteriophage  $\phi$ US3. It was described that the  $\phi$ vML3 lysin, which is active on a wide range of lactococcal strains, lacked homology with known lytic enzymes. The 25 bacteriophage  $\phi$ US3 was identified during studying bacteriophages specific for the cheese-making strain *Lactococcus lactis* SK11 (NIZO). The results showed that the deduced amino acid sequence of *lytA* shares similarities with that of an autolysin of *Streptococcus pneumonia*, suggesting that the bacteriophage  $\phi$ US3 encodes an amidase rather than a lysozyme-type 30 muramidase. The above illustrates the difficulties facing a person skilled in the art wishing to isolate DNA-sequences from different organisms. The lack of information regarding sequences and the lack of homology between known sequences makes use of probes and primers derived from known sequences quite unlikely to lead to successful isolation of a 35 correct DNA sequence encoding a holin from different organisms.

In EP-A2-0 510 907 (AFRC, M.J. Gasson, published 28 October 1992, ref. 7) the use of bacteriophages of food-contaminating or pathogenic bacteria or the lysins thereof to kill such bacteria was described. Examples included lysins from bacteriophages of *Listeria*

*monocytogenes* (phage  $\phi$ LM4) and *Clostridium tyrobutyricum* (phage  $\phi$ P). Also tests for bacterial contamination can be made specific for specific bacteria by using the appropriate bacteriophage or lysin thereof and determining whether cells are lysed thereby. That European patent thus describes the use of lysins obtained from phages of food-contaminating or even pathogenic bacteria, which is not desirable for food-grade applications. Moreover, the use of such lysins is further away from the subject of this invention, which will be discussed below as it does not lie in improving flavour of food products by autolysis of lactic acid bacteria.

In another aspect the invention relates to a process for inhibiting the growth of a culture of lactic acid bacteria without lysing the cells.

The growth of lactic acid bacteria can be inhibited in several ways.

For example, in normal fermentations with lactic acid bacteria, e.g. for the production of yoghurt, when a certain low pH is obtained the high amount of lactic acid stops further fermentation. The growth changes from the log phase to the stationary phase which in effect is some sort of inhibition of the growth.

Another possibility is that the nutrients become scarce and the so-called starvation occurs, because the necessary ingredients are no longer available for growth of the bacteria. This means that no further growth occurs.

Still another possibility is the effect of pasteurization or sterilization causing cell death.

#### Summary of the invention

It has now been found that holin on its own already has a bacteriostatic effect on Gramnegative bacteria like *E. coli* and Grampositive bacteria like lactic acid bacteria. Thus according to a first embodiment the invention provides a process as described in claim 1, i.e. a process for inhibiting the growth of a culture of lactic acid bacteria, which process comprises the *in situ* production in the cells of the lactic acid bacteria of a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria, the gene encoding said holin being under control of a first regulatable promoter, said first regulatable promoter not normally being associated with said holin gene, said holin being capable of exerting a

bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired.

According to a second embodiment the invention provides a process as described in claim 2, i.e. a process according to the first embodiment, which additionally comprises the *in situ* production in the cells of the lactic acid bacteria of a lysin obtainable from lactic acid bacteria other foodgrade grampositive microorganisms or their bacteriophages, the gene encoding said lysin being under control of a second regulatable promoter, whereby the produced lysin effects lysis of the cells of the grampositive or gramnegative bacteria, preferably the lactic acid bacteria.

Preferably the second regulatable promoter is the same as the first regulatable promoter (claim 3), and more preferably the gene encoding the holin and the gene encoding the lysin are placed under control of the same regulatable promoter in one operon (claim 4). It is advantageous for food fermentations when said first or second promoter or both can be regulated by food-grade ingredients or parameters (claim 5). The processes according to the invention can be used in the culture of lactic acid bacteria as such, but they can also be used in a product containing such culture (claim 6). A specific embodiment of this latter possibility is a process in which the lactic acid bacteria culture is used for producing a fermented food product obtainable by the fermentative action of the lactic acid bacteria and subsequently the lactic acid bacteria in the fermented food product are lysed (claim 7). A specific example of such process is one in which the fermented food product is a cheese product (claim 8). Then an additional cheese ripening step can be carried out, whereby some of the constituents after leaving the lysed cells will change the composition of the cheese product (claim 9).

A third embodiment of the invention relates to a process for combatting spoiling bacteria or pathogenic bacteria, in which a lysed culture obtained by a process according to the second embodiment of the invention is used as a bactericidal agent (claim 10). One way of use as a bactericidal agent is a process for improving the shelf life of a consumer product, in which a product obtained by a process according to either the first or the second embodiment of the invention and containing free holin or free lysin or both is incorporated into said consumer product in such amount that in the resulting consumer product the growth



of spoiling bacteria or pathogenic bacteria is inhibited or that their viability is strongly reduced (claim 11). Such consumer products comprise edible products, cosmetic products, and products for cleaning fabrics, hard surfaces and human skin (claim 12).

5           Examples of such products may be bread and bread improvers; butter, margarine and low calorie substitutes therefor; cheeses; dressings and mayonnaise-like products; meat products; food ingredients containing peptides; shampoos; creams or lotions for treatment of the human skin; soap and soap-replacement products; washing powders or  
10           liquids; and products for cleaning food production equipment and kitchen utensils.

          A fourth embodiment of the invention is a process for modifying a mixture of peptides, which comprises (1) combining a culture of lactic acid bacteria with a mixture of peptides obtained by proteolysis of  
15           proteins, the cells of said culture containing both a gene encoding a holin under control of a first regulatable promoter and a gene encoding a lysin under control of a second regulatable promoter, which second and first promoter can be the same and which first and second promoter are not normally associated with the respective genes, and (2) effecting  
20           induction of the promoter or promoters for producing both the holin and the lysin in such amounts that the cells of the lactic acid bacteria are lysed and the contents of the cells containing peptidases will modify the composition of the mixture of peptides (claim 13). In order to achieve sufficient bacterium growth the host cell must not lyse too quickly,  
25           preferably lysis will occur at the end of the log phase or commencement of the stationary phase.

          An alternative is a process for modifying a mixture of peptides, which comprises treating a mixture of peptides obtained by proteolysis of proteins with a lysed culture obtained by a process  
30           according to the second embodiment of the invention (claim 14).

          The proteins to be proteolysed can be, for example, milk proteins or vegetable proteins, or both (claim 15).

          Any of the above-mentioned processes as claimed in claims 1-15, wherein the holin is encoded by a nucleic acid sequence according to any  
35           of claims 18-20 and/or is expressed from a recombinant vector according to any of claims 21-24 and/or is expressed by a recombinant cell according to any of claims 25-27 fall within the intended scope of the invention. In addition an alternative suitable embodiment of a process according to the invention can be directed at the inducible expression of

a lysin having the amino acid sequence of sequence id no 7 or being a functional equivalent thereof.

5 A nucleic acid sequence encoding a holin derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from such a grampositive bacterium also falls within the scope of the invention. Such a nucleic acid sequence can for example encode the amino acid sequence of sequence id no 6 or a functional equivalent thereof such as the nucleic acid sequence of nucleotides 103-328 of sequence id no 5. Any nucleic acid sequence  
10 according to the invention can further be operatively linked to a first regulatable promoter, said first regulatable promoter not normally being associated with the holin encoding sequence.

Also comprised by the invention are recombinant vectors comprising any of the nucleic acid sequences in any of the claimed  
15 embodiments, said vector preferably further being foodgrade. In addition such a recombinant vector according to the invention may suitably further comprise a nucleic acid sequence encoding a lysin, both the holin and the lysin being derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a L. lactis or a bacteriophage derivable from  
20 such a grampositive bacterium. a preferred embodiment of a recombinant vector according to the invention further comprises the natural attachment/integration system of a bacteriophage. The natural attachment/integration system of a bacteriophage can comprise the bacteriophage attachment site and an integrase gene located such that  
25 integration of the holin and optionally lysin gene will occur, said system preferably being derived from a bacteriophage that is derivable from a food grade host cell, preferably a lactic acid bacterium. A suitable recombinant vector according to the invention comprises the nucleic acid sequence encoding the holin and the nucleic acid sequence  
30 encoding the lysin operatively linked to a foodgrade inducible promoter that can be induced via a food grade mechanism. Such a promoter system can for example be a thermosensitive complex inducible promoter as is disclosed in EP94201355 and is present on plasmid pIR14. A recombinant host cell comprising a nucleic acid sequence according to any of claims  
35 18-20 in a setting other than in its native bacteriophage and/or a recombinant vector according to any of claims 21-24 is claimed. Any of the abovementioned embodiments of recombinant host cell further comprising a nucleic acid sequence encoding a lysin, said lysin preferably being derivable from a grampositive bacterium such as a lactic

acid bacterium, in particular a *L. lactis* or a bacteriophage derivable from such a grampositive bacterium, said nucleic acid sequence encoding a lysin preferably being in a setting other than in its native bacteriophage or bacterium is also suitable. Preferably a recombinant host cell according to the invention will be a food grade host cell, preferably a lactic acid bacterium. Most preferably the host cell is of the same type from which the holin and/or lysin encoding nucleic acid sequences are derived.

#### 10. Brief description of the drawings

##### FIGURE LEGENDS belonging to the draft publication

Fig. 1. A) Schematic outline of the PCR reactions used for the amplification of *lytP*, *lytR*, and the combination of *lytP* and *lytR*. The ORF's are indicated by hatched arrows. Sequences of the amplification primers 1-4 (*lyt1-lyt4*) are given in Table 2 and as sequence id no 1-4 in the Sequence Listing. The scale is in kilobases (kb).

B) Schematic representation of the plasmid constructions. See for details Materials and Methods. Abbreviations:  $Em^R$ , erythromycin resistance marker;  $Amp^R$ , ampicillin resistance marker;  $Cm^R$ , chloramphenicol resistance marker;  $P_{spac}$  is a hybrid regulatory region, constructed by Yansura and Henner (29), which contains the RNA polymerase recognition sequences of an early SP01 promoter and the *lac* operator; *lacI*, *lac* repressor under the control of the *Bacillus licheniformis* penicillinase transcriptional and translational signals, indicated as  $P_{pen}$  (29);  $P_1$  and  $P_2$ , promoters  $P_1$  and  $P_2$  of the bacteriophage R1-t; T, transcription terminator; ori, origin of replication; *rro*, R1-t repressor gene.

Fig. 2. Alignment of ORF 23 and the *L. lactis* subsp. *cremoris* c2 lysin (c2). Identical amino acid residues are indicated with asterisks, conserved changes by dots.

Fig. 3. Nucleotide sequence of a 1200 bp DNA fragment of R1-t carrying *lytP* and *lytR* as represented in sequence id no 5. The deduced amino acid sequences of *lytP* and *lytR* are indicated in sequence id no 6 and 7 respectively. The putative ribosomal binding sites (RBS) are underlined. Asterisks represent stop codons. The stem-loop structure downstream of *lytR* is indicated by solid arrows.

Fig. 4. Analysis of the lytic activity of the *lytR* gene product. Cell free extracts of *E. coli* cells carrying the plasmid pAG58 (lanes 1 and 2)

or pAG58R (lanes 3 and 4) were obtained two hours after the addition of IPTG. Abbreviations: ni, non-induced; i, induced. The arrow indicates the position of a clearing zone as a result of lytic activity exhibited by the *lytR* gene product.

- 5 Fig. 5. A) Deduced amino acid sequence of the *lytP* gene product (sequence id no 6). Predicted transmembrane segments are indicated by bars, the predicted  $\beta$ -turn region by t's. Charged amino acid residues are indicated + or -, depending on the sign of the charge.

- 10 B) Topological model of *LytP* based on the computer predictions. The membrane-spanning amino acids are indicated.

- 15 Fig. 6. A) The effect of expression of *lytP*, *lytR*, or the combination of *lytP* and *lytR* on the optical density of *E. coli* MC1000 cells. Optical density measurements of *E. coli* cells carrying either pAG58 (a), pAG58R (b), pAG58P (c) or pAG58PR (d), with (•) or without (◦) the addition of the inducer (IPTG) are indicated as a function of time. The time scale is in hours before and after the time of induction (indicated by arrow).

- 20 B) The number of colony forming units per ml of *E. coli* cells carrying either pAG58 (empty bars), pAG58R (checkered bars), pAG58P (hatched bars), or pAG58PR (filled bars) before (left diagram), and after two hours after induction (right diagram).

- 25 Fig. 7. The effect of the induced expression of *lytP*, *lytR*, or the combination of *lytP* and *lytR* on the optical density of *L. lactis* subsp. *cremoris* LL302 cells. Optical density measurements of induced *L. lactis* cells carrying either pIR12 (•), pIR1P (Δ), pIR1R (▲), or pIR1PR (◻) respectively, are indicated as a function of growth. The optical density (OD) measurements of *L. lactis* carrying pIR1PR, not exposed to mitomycin C, are represented by (◦). Time scale is in hours after the time of induction with mitomycin C (1  $\mu$ g/ml).

- 30 The invention is illustrated by a draft publication, which is given below.

- 35 Inducible lysis of *Lactococcus lactis* mediated by the *Lactococcus lactis* subsp. *cremoris* bacteriophage R1-t lysis functions.

#### SUMMARY

This work describes the involvement of two genes of the temperate *Lactococcus lactis* subsp. *cremoris* bacteriophage R1-t, *lytR* and

*lytP*, in the lysis of its host. The gene product of *lytR* exhibits lytic activity as it hydrolysed *Micrococcus lysodeikticus* autoclaved cell walls. The gene product of *lytP* is required in conjunction with *lytR* to obtain efficient lysis *in vivo* in *Escherichia coli* as was shown by induction studies monitoring the optical density as a measure of cell lysis: expression of *lytR* alone did not cause significant lysis of *E. coli* cells whereas simultaneous expression of *lytP* and *lytR* caused lysis of this bacterium. *lytP* therefore seems to have a similar function as the S protein of the *E. coli* phage lambda, *i.e.* rendering the murein substrate accessible to the lysin.

Both *lytP* and *lytR* were subcloned in an inducible expression-vector for *L. lactis*. Induction of both genes in *L. lactis* was shown to result in cell lysis as monitored by a decrease in optical density.

## 15 INTRODUCTION

Host cell lysis by temperate bacteriophages is accomplished by at least two fundamentally different mechanisms (30). The small single-stranded DNA phage  $\phi$ X174 encodes a protein which forms a channel to transport complete phage particles from the cytoplasm of the host to the environment (4, 7, 27). However, most of the known phages encode an enzyme with murein-degrading activity. These so-called lysins cause breakdown of the peptidoglycan layer which is followed by lysis of the host and the release of the phage particles.

Lysins of bacteriophages of Gram-negative bacteria so-far characterized lack a signal sequence needed for *sec*-dependent transport across the inner membrane. A second lysis function, encoded by a gene located immediately upstream of the lysin gene, is required for efficient lysis. This gene encodes a so-called holin which is believed to form holes in the cell membrane, thereby rendering the murein substrate accessible to the lysin (30).

Until recently it was believed that in Gram-positive bacteria, phage-mediated lysis was solely accomplished through the action of a phage-encoded lysin. Transit of the phage-encoded lysin across the membrane was thought to occur via the general secretory route. However, the observation that a signal sequence required for this type of transport is absent in many of the identified lysins, raised the question how the murein-degrading activity gains access to the cell wall. There is now growing support for the idea that many of these phages require a second function for lysis of their Gram-positive host. Recently it was

shown that the *Bacillus subtilis* phage  $\phi$ 29, gene 14, situated immediately upstream of the lysin gene, specifies a protein required for efficient release of the phage lysin to the substrate-containing environment (20). Sequence analysis of the complete *L. lactis* subsp. *cremoris* bacteriophage R1-t genome has revealed the presence of an open reading frame (ORF) similar to several lysins of other bacteriophages. In this work we show that the corresponding gene, designated *lytR*, indeed encodes a protein with cell wall degrading activity *in vitro*.

Table 1. Bacterial strains, plasmids and bacteriophage

	relevant features	reference
<b>Bacterial strains</b>		
<i>L. lactis</i> subsp. <i>cremoris</i>		
LL302	MG1363 carrying the pWV01 <i>repA</i> gene on the chromosome to ensure efficient replication	This work
<i>E. coli</i>		
MC1000	<i>araD</i> 139, $\Delta$ <i>lacx</i> 74, $\Delta$ ( <i>ara</i> , <i>leu</i> )7697, <i>galU</i> , <i>galK</i> , <i>strA</i>	3
<b>Plasmids</b>		
pUC18	Ap <sup>r</sup>	28
pXNB	Ap <sup>r</sup> ; pUC18 derivative, containing a 4.1-kb <i>Xba</i> I/ <i>Nhe</i> I-fragment of phage R1-t	This work
pAG58	Ap <sup>r</sup> ; Cm <sup>r</sup>	8
pAG58P	Ap <sup>r</sup> ; Cm <sup>r</sup> ; pAG58 derivative carrying <i>lytP</i>	This work
pAG58R	Ap <sup>r</sup> ; Cm <sup>r</sup> ; pAG58 derivative carrying <i>lytR</i>	This work
pAG58PR	Em <sup>r</sup> ; Cm <sup>r</sup> ; pAG58 derivative carrying <i>lytP</i> and <i>lytR</i>	This work
pUC18P	Ap <sup>r</sup> ; pUC18 derivative carrying <i>lytP</i>	This work
pUC18R	Ap <sup>r</sup> ; pUC18 derivative carrying <i>lytR</i>	This work
pUC18PR	Ap <sup>r</sup> ; pUC18 derivative carrying <i>lytP</i> and <i>lytR</i>	This work
pIR12	Em <sup>r</sup> ; carrying the regulatory region of R1-t	This work
pIR1P	Em <sup>r</sup> ; pIR12 derivative carrying <i>lytP</i>	This work
pIR1R	Em <sup>r</sup> ; pIR12 derivative carrying <i>lytR</i>	This work
pIR1PR	Em <sup>r</sup> ; pIR12 derivative carrying <i>lytP</i> and <i>lytR</i>	This work
<b>Bacteriophage</b>		
R1-t	type P335, small isometric lactococcal phage, isolated from <i>L. lactis</i> subsp. <i>cremoris</i> R1	9, 11
Em <sup>r</sup> , Ap <sup>r</sup> , and Cm <sup>r</sup> represent resistances to erythromycin, ampicillin, and chloramphenicol, respectively.		

With the use of two species-specific inducible expression systems we show that *LytR* requires an additional gene product, specified by *lytP* upstream of *lytR*, for efficient *in vivo* lysis of *E. coli* and *L. lactis*.

## MATERIALS AND METHODS

### Bacterial strains, phage, plasmids, and media

The bacterial strains, phage and plasmids used in this study are listed in Table 1.

*E. coli* was grown in TY broth (17) or on TY broth solidified with 1.5% agar. *L. lactis* was grown in glucose M17 broth (21), or on glucose M17 agar. Erythromycin was used at 100 µg/ml and 5 µg/ml for *E. coli* and *L. lactis*, respectively. For *E. coli*, ampicillin and chloramphenicol were used at a concentration of 100 µg/ml and 5 µg/ml, respectively.

### DNA techniques

Plasmid DNA was isolated essentially by the method of Birnboim and Doly (1). Restriction enzymes, Klenow enzyme, T4 DNA ligase, and T4 DNA polymerase were obtained from Boehringer GmbH (Mannheim, Germany) and used according to the instructions of the supplier. Synthetic oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). Polymerase chain reactions were performed using Vent polymerase (New England Biolabs Inc., Beverly, MA.). Samples were heated to 94 °C for 2 min, after which target DNA was amplified in 25 subsequent cycles under the following conditions: 94 °C for 1 min; 50 °C for 2 min; 73 °C for 1 min. The primers used for amplification are listed in Table 2 and sequence id no 1-4 of the Sequence Listing. *E. coli* was used as a host for obtaining recombinant plasmids. Transformation of *E. coli* was performed by the method of Mandel and Higa (12). Plasmids were introduced in *L. lactis* subsp. *cremoris* LL302, which contains a copy of the pWV01 *repA* gene on the chromosome to ensure efficient replication, by means of electroporation (23). DNA and protein sequences were analyzed using the programs developed by Staden (19). Analysis of the *LytP* protein was computed with the PC/Gene program (version 6.7; IntelliGenetics, Inc., Geneva, Switzerland) using the membrane spanning domain search program SOAP, or the  $\beta$ -turn search program BETATURN.

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 Table 2. Primers used for amplification of *lytP* and *lytR*


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primer	DNA sequence (5'→3')
Lyt1	AAAACCCGGGAAGCTTGTGACAGCAGTGATTGGTTCAACG
Lyt2	TTCTAGAAGCTTGCATGCCCTTCTTTTTATTATTGAC
Lyt3	AAAACCCGGGAAGCTTGTGACGATAATACAGCAAGCCTAGTC
Lyt4	TTTTTCTAGAAGCTTGCATGCCAAGCGGGTTAATTTATCC

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*Hind*III (AAGCTT), *Sph*I (GCATGC), and *Sal*I (GTCGAC) restriction enzyme sites are depicted boldfaced.

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#### IPTG and mitomycin C induction

Overnight cultures were diluted hundred-fold in fresh glucose M17 medium (*L. lactis*) or TY medium supplemented with 0.5% glucose (*E. coli*) and grown until the culture reached an OD600 of 0.3 at which point isopropyl-β-D-thiogalactopyranoside (IPTG) or mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 5mM or 1 μg/ml, respectively. Before the addition of IPTG, *E. coli* cells were collected by centrifugation and resuspended in an equal volume of TY medium without additional glucose.

#### Lytic activity assay

The lytic activity assay was performed essentially as described by Potvin *et al.* (15) with some minor adjustments as reported by Buist *et al.* (2).

#### Plasmid constructions

The *lytP* and *lytR* containing fragments of the R1-t genome were amplified using polymerase chain reactions (PCR's). A 4.1-kb *Xba*I/*Nhe*I fragment of the R1-t genome containing both *lytP* and *lytR* was subcloned in the unique *Xba*I site of pUC18 resulting in the plasmid pXNB. Using pXNB as a template, amplification with three different primer combinations (*lyt1-lyt2*, *lyt3-lyt4*, and *lyt1-lyt4*; see Fig. 1A) yielded three DNA fragments carrying either *lytP*, *lytR*, or the combined *lytP* and *lytR*, respectively. Following digestion with *Sph*I and *Hind*III these three PCR products were subcloned in *Hind*III and *Sph*I restricted pAG58, which



resulted in plasmids pAG58P, pAG58R, and pAG58PR, carrying *lytP*, *lytR*, and the combined *lytP* and *lytR*, respectively, under the control of the IPTG inducible  $P_{\text{spac}}$  promoter (Fig. 1B). For lysis studies in *L. lactis* plasmids pAG58P, pAG58R and pAG58PR were first restricted with *SphI* and *SalI*. Subsequently, the DNA fragments, carrying *lytP*, *lytR* and the combination of *lytP* and *lytR*, were first subcloned in *SphI/SalI*-cut pUC18. The *HindII/HindIII* fragments of these three constructs, designated pUC18P, pUC18R, and pUC18PR, were cloned in the *NruI* and *HindIII* sites of pIR12, resulting in the plasmids pIR1P, pIR1R, and pIR1PR, containing *lytP*, *lytR*, and both *lytP* and *lytR*, respectively, under the transcriptional control of the R1-t promoter-operator region (Fig. 4B). These plasmids were transformed to *L. lactis* subsp. *lactis* strain LL302 which contains a copy of the pWV01 *repA* gene on the chromosome to ensure efficient replication of pWV01-derived plasmids.

The construction of plasmid pIR12 was described in a co-pending application EP-94201353.3, filed on the same date entitled: Process for the lysis of a culture of lactic acid bacteria by means of a lysin, and uses of the resulting lysed culture, the specification of which is incorporated herein by reference.

## RESULTS

### Cloning and sequence analysis of the R1-t lysis functions.

The switch from lysogenic to lytic life cycle of the temperate *L. lactis* subsp. *cremoris* phage R1-t will ultimately result in host cell lysis caused by phage-encoded lysis function(s), followed by the release of phage particles. Inspection of the DNA sequence of R1-t revealed that ORF 23, which specifies a protein of 270 amino acids with a calculated molecular weight of 30,214 Da, shows significant similarity with the lysin genes of the *L. lactis* bacteriophages c2 (sequence id no 8) and  $\phi$ ML3 (26, 18). The similarity between the deduced amino acid sequence of ORF 23 with the c2 lysin is shown in Figure 2. Moreover, ORF 23 specifies an amino acid sequence similar to the amino acid sequences of the N-terminal portions of the amidase Hbl of the *Streptococcus pneumoniae* bacteriophage HB-3 (16), and the *S. pneumoniae* LytA autolysin (5). Therefore, ORF 23, hereafter designated *lytR* (Fig. 3) (sequence id no 7), is a likely candidate for the phage-encoded lysin gene. This was not to be predicted as is apparent from the previously cited Young reference.

To test this supposition, *lytR* was cloned into the IPTG inducible expression-vector pAG58, resulting in pAG58R (Fig. 1B). Cell-

free extracts of *E. coli* cells containing pAG58R were assayed for lytic activity on an SDS-polyacrylamide gel in which *Micrococcus lysodeikticus* autoclaved cell walls were co-polymerized. After staining of the cell wall-containing gel with methylene blue, a clearing zone is expected at positions corresponding to lytic proteins due to the breakdown of incorporated cell walls. As shown in Figure 4, in cell free extracts of pAG58-containing *E. coli* cells a clearing zone was absent at the position corresponding to a protein with the expected molecular weight of the *lytR*-encoded protein. Cell free extracts of pAG58R-containing cells, however, gave rise to a clearing zone at the expected position. A weak clearing zone was obtained with cell free extracts of uninduced cells due to limited expression of the *lytR* gene. Cell free extracts of induced pAG58R-containing cells showed an extended clearing zone, which became very large in extracts obtained two hours after induction.

According to the rules of Von Heijne (25), *LytR* does not seem to contain a signal sequence specific for secreted proteins using the *sec*-dependent transport system. This apparent lack of a signal sequence has also been observed in lysins of other bacteriophages of both Gram-negative and Gram-positive bacteria. For host cell lysis to occur these phages require a protein that forms holes in the cytoplasmic membrane to render the host cell peptidoglycan layer accessible to the lysin (30). ORF 22, which is situated upstream of *lytR*, specifies a protein of 75 amino acids with a predicted molecular weight of 7,688 Da (sequence id no 6). Although the predicted amino acid sequence shows no similarity with the putative hole-forming proteins of other phages, computer analysis of the protein product of ORF 22, designated hereafter as *lytP*, predicted structural similarities with these proteins. Computer analysis revealed that the protein, specified by *lytP*, has a high probability of containing a pair of transmembrane domains, separated by a sequence with a high probability of adopting a beta turn conformation (Fig. 5). In addition it contains a charged C terminus and is highly hydrophobic. Therefore, this protein might function as a pore-forming protein required for the release across the cytoplasmic membrane of the R1-t encoded *LytR*.

### *LytP* and *lytR* are required for lysis in *Escherichia coli*

To determine whether the *lytP* and *lytR* gene products are involved in host cell lysis, *lytP*, *lytR*, and the combination of *lytP* and *lytR* were subcloned in the inducible expression- vector pAG58, resulting in pAG58P, pAG58R and pAG58PR, respectively (Fig. 1B). Induction studies

were performed with *E. coli* MC1000 carrying these plasmids to examine the effects of the expression of the cloned genes on the optical density of the cells (Fig. 6A). Induction of *lytR* expression did not cause any lysis of pAG58R-containing *E. coli* cells as was determined by optical density measurements. The induction of *lytP* expression, however, almost immediately halted the increase in the optical density of pAG58P-containing cells. The expression of both *lytP* and *lytR* in *E. coli* caused lysis. Lysis occurred almost immediately after the addition of IPTG to pAG58PR-containing cells, as was demonstrated by the decrease in optical density which was associated with a dramatic decrease in colony forming units (CFU's) as compared to the uninduced control (Fig. 6B). No significant difference in CFU's between cells carrying pAG58 and pAG58R was observed. However, the induction of *lytP* had a significant effect on the viability of pAG58P-containing cells. The number of CFU's dropped more than 200-fold within two hours.

#### Expression of *lytP* and *lytR* in *Lactococcus lactis*

In order to examine the effects of the expression of either *lytP*, *lytR* or the combined *lytP* and *lytR* on the optical density of *L. lactis* cells, plasmids pIR1P, pIR1R, and pIR1PR were constructed (Fig. 1B). Transcription of *lytP*, *lytR*, and both *lytP* and *lytR* in these plasmids is controlled by the regulatory region of phage R1-t, which incorporates the gene specifying the repressor (*rro*) of R1-t in addition to its cognate operator region (see Fig. 1B). Expression was induced by the addition of the DNA damaging substance mitomycin C. Induction studies were performed with *L. lactis* subsp. *cremoris* LL302 cells carrying the plasmids described above. Figure 7 shows that the addition of mitomycin C to *L. lactis* cells carrying pIR12 slows down the increase in optical density similar to pIR1P-containing *L. lactis* cells. The addition of mitomycin C to genetically non-modified lactococci caused lysis of a small portion of the cells (results not shown). The expression of *lytR* as well as the simultaneous expression of *lytP* and *lytR* led to a decrease in optical density, as compared to pIR12-containing cells to which mitomycin C had been added, indicating cell lysis.

#### DISCUSSION

We recently determined the nucleotide sequence of the temperate *L. lactis* subsp. *cremoris* bacteriophage R1-t. On the basis of the similarity of the deduced amino acid sequence with various (auto)lysins

we postulated that ORF 23, designated *lytR*, could specify the phage-encoded lysin. The *lytR* gene product would consist of 270 amino acids with an estimated molecular weight of 30,214 Da. By assaying cell-free extracts of *E. coli* cells expressing *lytR*, it was shown that *lytR* indeed  
5 specified a protein with lytic activity.

The similarity of LytR is mainly limited to the C-terminal parts of the lysins of the lactococcal bacteriophages c2 and  $\phi$ vML3, whereas the N-terminal part of LytR is similar to the amino acid sequence of the N-terminal portion of the *S. pneumoniae* LytA autolysin. It has  
10 been proposed that LytA consists of two functional modules (16), the C-terminal domain specifying the binding site to the murein substrate and the N-terminal domain determining the specificity of the enzyme. Since LytA is an *N*-acetylmuramoyl-L-alanine amidase (6), it is tempting to speculate that LytR is also an *N*-acetylmuramoyl-L-alanine amidase.

15 Because of the lack of an apparent signal peptide, we hypothesized that, like many other phage-encoded lysins, LytR needs an additional factor in order to gain access to the cell wall. ORF 22, designated *lytP*, which is situated immediately upstream of *lytR*, can specify a protein of 75 amino acids with the characteristics of a so-called holin which, for other phages, was shown to render the murein  
20 substrate accessible to lysins which lack a signal peptide (30).

This hypothesis was corroborated by the observation that the expression of *lytP* is indeed needed for efficient lysis of *E. coli* *in vivo*. In fact, induction of *lytR* expression did not result in lysis of *E.*  
25 *coli*. However, *E. coli* did lyse when, in addition to *lytR*, *lytP* was also expressed. From these results it was concluded that the transit of LytR across the inner membrane is dependent on the *lytP* gene product. The induction of solely *lytP* almost immediately halted the increase in optical density and had a dramatic effect on the viability of the induced  
30 cells. This is probably caused by the spontaneous insertion of the protein into the lipid bilayer, inducing nonspecific lesions in the inner membrane and thereby dissipating the membrane potential (20). Presumably LytP forms pores in the cytoplasmic membrane, thus allowing LytR to gain access to the cell wall.

35 An inducible expression system for Lactococci recently developed in our laboratory, made it possible to examine the effects of expression of *lytP*, *lytR*, and the combined *lytP* and *lytR* in *L. lactis*. Expression of the combined *lytP* and *lytR* in *L. lactis* resulted in lysis of the cells. In contrast to *E. coli*, lysis was also observed when only

*lytR* was expressed. Lysis of cells solely expressing *lytR* is probably caused by the combined effect of mitomycin C and *LytR*: Since mitomycin C lyses a small proportion of the cells (results not shown), *LytR* is extruded in the medium, thus acting upon the cell wall from without, and masking the additional requirement for *LytP* to effect lysis as was the case in *E. coli*.

For bacteriophages of both Gram-negative and Gram-positive bacteria, a system based on a murein hydrolase and a second protein required for the access of the hydrolase to its murein substrate, seems to be a general phenomenon in lysis strategies. Recently it was shown that, in addition to the *B. subtilis* phage  $\phi 29$ -encoded lysin, efficient lysis of *E. coli* also required the gene 14 product. Also several lactococcal bacteriophages seem to encode an additional factor needed for host cell lysis. On the basis of structural similarity, it has been postulated that the bacteriophages c2 and  $\phi vML3$  encode a holin (26, 30). The deduced amino acid sequence of ORF2 of the virulent bacteriophage  $\phi U53$ , isolated from *L. lactis* SK11 (14), also shares the characteristic structural traits of a holin, making it likely that it is involved in the translation of the phage-encoded lysin, *LytA*. This report, however, proves for the first time that a *Lactococcus*-specified holin is required for phage-induced lysis.

#### DRAFT PUBLICATION 2

Development of a food-grade, thermo-inducible lysis system using the regulatory region and lysis functions of the temperate *Lactococcus lactis* subsp. *cremoris* bacteriophage R1-t.

#### Introduction

The system is based on the food-grade removal of most of the genomic DNA of a temperate lactococcal bacteriophage in such a way that an inducible regulatory region of the temperate bacteriophage is directly placed upstream of the lysis functions encoded by the prophage. As an example of the general applicability of this strategy to any prophage with a similar genetic structure, bacteriophage R1-t was taken. To obtain the desired deletion, plasmid pBTS1 was constructed (Figure 8). In the future pBTS2 will be constructed in which *rro* is replaced by *rro*<sup>TS</sup> and therefore can be used to make this system thermo-inducible.

## Experimental procedures

### *Bacterial strains, phage, plasmids, and media*

5           The bacterial strains, phage, and plasmids used in this study  
are listed in Table 3. *Escherichia coli* JM101 was grown in TY broth  
(Rottlander and Trautner, 1970) with vigorous agitation, or on TY agar,  
at 37 °C. When needed, ampicillin, isopropyl- $\beta$ -D-thiogalactopyranoside  
(IPTG) and 5-bromo-4-chloro-3-indolyl  $\beta$ -galactopyranoside (X-gal) (all  
10       from Sigma Chemical Co., St. Louis, MO.) were used at concentrations of  
100  $\mu$ g/ml, 1mM and 0.002% (wt/vol), respectively. *L. lactis* subsp.  
*cremoris* was grown in M17 broth (Terzaghi and Sandine, 1975), or on M17  
agar, supplemented with 0.5% glucose or lactose at 30 °C. When  
appropriate, erythromycin (Boehringer Mannheim, GmbH, Germany) and X-gal  
15       were used at concentrations of 5  $\mu$ g/ml and 0.004% (wt/vol), respectively.

### *General DNA techniques and transformation*

          General DNA techniques were performed as described by Sambrook  
20       *et al.* (1989). Plasmid DNA was isolated by the method of Birnboim and  
Doly (1979) and by using QIAGEN Midi-Plasmid isolation columns (Qiagen  
Inc., Chatsworth, Ca.). Restriction enzymes, alkaline phosphatase and T4  
DNA ligase were obtained from Boehringer Mannheim and were used according  
to the instructions of the supplier. Transformation of *E. coli* was  
25       performed as described by Mandel and Higa (1970). *L. lactis* LL108 was  
transformed by electroporation using a Gene Pulser (Bio-Rad Laboratories,  
Richmond, Calif.), as described by Holo and Nes (1989) with the  
modifications suggested by Leenhouts and Venema (1993). Electroporation  
of *L. lactis* R1, R131 and R1K10 was done as described by van der Lelie *et*  
30       *al.* (1988). Oligonucleotides were synthesized using an Applied Biosystems  
381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).  
Polymerase chain reactions (PCR's) were performed using Vent polymerase  
(New England Biolabs, Inc., Beverly, MA.). After heating of the samples  
to 94 °C for two minutes, target DNA was amplified in 30 subsequent  
35       cycles under the following conditions: 94 °C for 1 min; 50 °C for 2 min;  
73 °C for 3 min. PCR fragments were purified using the QIAEX DNA Gel  
Extraction Kit (Qiagen Inc.).

*Isolation of R1-t phage particles and DNA*

An overnight culture of *L. lactis* R1 was diluted hundred-fold in 500 ml fresh lactose M17 medium and grown until the culture reached an OD<sub>600</sub> of 0.8 at which point mitomycin C (Sigma) was added to a final concentration of 2.5 µg/ml. Incubation at 30 °C was continued in the dark until lysis occurred. Cells debris was removed by centrifugation for 10 min at 6000 rpm. Phage particles were precipitated by incubation with NaCl (0.5 M) and polyethylene glycol 6000 (10 % [wt/vol]) for three hours on ice and purified by a CsCl step gradient as described by Sambrook *et al.* (1989). The bacteriophage R1-t suspension was dialysed against several changes of 150 mM NaCl, 15 mM trisodiumcitrate. Phage DNA was obtained by extracting the suspension twice with phenol. The DNA solution was subsequently dialysed against 10 mM Tris-HCl/1 mM EDTA, pH 8.0.

*Sequencing attB-sites*

The attachment sites *attL* and *attR* of the bacteriophage R1-t lysogen *L. lactis* R1 were determined by means of cycle sequencing using the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent (*exo*<sup>-</sup>) DNA Polymerase (Biolabs, New England). Primers *attBL* and *attBR* with flanking *XbaI* and *PstI* sites (Table 4) were used for cloning the *attB* site of *L. lactis* MG1363. The 272-bp PCR fragment obtained with *attBL* and *attBR* was cut with *XbaI* and *PstI* and cloned in the *XbaI/PstI* sites of pUC18 and sequenced using the dideoxy-chain-termination method (Sanger *et al.*, 1977) and the T7 sequencing kit (Pharmacia AB, Uppsala, Sweden).

*Phage titre determination*

Supernatant taken from *L. lactis* R131 was diluted in 1 mM MgSO<sub>4</sub>. The indicator strain *L. lactis* R1K10 was grown in GM17 until the OD<sub>600</sub> was 0.7. 2 ml of culture were centrifuged and cells were resuspended in 2 ml 1 mM MgSO<sub>4</sub>. An aliquot of 100 µl diluted phage-particles were added to 200 µl cells. After incubation at room temperature for 20 minutes 3 ml Top agar (0.7% GM17 agar, 0.25% glycine, 10 mM CaCl<sub>2</sub>) were added, mixed, and poored on a GM17 agar-plate (1.5%) containing glycine (0.25%) and CaCl<sub>2</sub> (10 mM). The plates were incubated overnight at 30 °C and the number of plaques were determined.

*Relysogenisation of L. lactis R1K10*

After infection of *L. lactis* R1K10 with R1-t phage particles, turbid plaques will be picked and tested for their ability to give UV-induction of prophage. Centrifuged cells of exponentially growing cultures will be resuspended in 1 ml 1 mM MgSO<sub>4</sub> and irradiated with a Mineralight u.v. lamp (model UVG-54, 254 nm, 3.2 Jm-2s-1: Ultra-violet Products Inc.) for 10 seconds, then 1 ml 2 times GM17 + 10 mM CaCl<sub>2</sub> will be added. The culture will be incubated at 30 °C until lysis occurs.

*Mitomycin C induction*

Overnight cultures of *L. lactis* were diluted hundred-fold in fresh glucose M17 medium and grown until an OD600 of 0.3 at which point mitomycin C was added to a final concentration of 1 µg/ml.

*Plasmid constructions*

The plasmid pORR1PR was constructed in *L. lactis* by subcloning the 2864-bp *EcoRI/SphI*-fragment of pIR1PR into pORI280 restricted with *EcoRI* and *SphI* (Figure 8). Homology analysis showed that ORF 25 of the R1-t genome shared 98% identity with the integrase-gene of bacteriophage phi LC3 (Lillehaug and Birkeland, 1993) and was therefore called *intR*. The *intR* region was amplified with flanking *SacI* and *XbaI* sites using PCR and primers int1 and int2 (Table 4). Plasmid pUC18Int was constructed by cloning the resulting 1326-bp PCR-fragment digested with *SacI* and *XbaI* into the *SacI/XbaI* sites of pUC18. A 1047-bp *HindIII* fragment of pUC18Int, which contains the 5'-truncated *intR*, was subcloned into the alkaline phosphatase-treated *SmaI*-site of pUC18. Both the resulting plasmid pUC18Intd and pUC18Int were constructed in *E. coli* JM101 (Figure 9). The 5'-truncated *intR* was cut out of pUC18Intd with *EcoRI* and *BamHI* and subcloned in the *EcoRI* and *BamHI* sites of pORR1PR, resulting in pBTS1 (Figure 8). The latter construction was done in *L. lactis* LL108.

In the future *rro* will be replaced by *rro*<sup>TS</sup> when this temperature inducible repressor is available. This will be done by replacing the 946-bp *NcoI-EcoRI* fragment of pBTS1 with the comparable fragment containing *rro*<sup>TS</sup>. This will result in pBTS2. Plasmid pIR14 deposited at the Centraal Bureau voor Schimmelcultures in Baarn. The



Netherlands comprises such a temperature sensitive *rro*. A detailed description is given in European Patent Application 94201355.8.

## Results

5

Plasmid pBTS1 was introduced in *L. lactis* LL108. As can be seen in figure 10, pBTS1 is still able to give inducible lysis after mitomycin C induction.

10

The initial idea was to introduce pBTS1 in *L. lactis* R1. As pBTS1 cannot replicate in *L. lactis* (it lacks the gene for the plasmid replication protein RepA) it will integrate into the chromosome of R1 under selective conditions. The integration will take place at either of three homologous regions: the *intR*-region (A), the regulatory-region (B) or the region of the lytic functions (C) (Figure 11). With appropriate primer-sets the place of integration can be distinguished (Table 5 and Figure 11). After the first recombination step in the regions A or C, a second recombination step in the region C or A, respectively, will delete the whole prophage and plasmid from the chromosome of strain R1, except for the desired functions. These two recombination steps will place the lytic functions directly under control of the regulatory region of R1-t, in a one copy situation at a well defined and stable place in the chromosome of *L. lactis*. If the integration takes place in region B (regulatory region), the second recombination step will not result in the substitution of *intR* and *rro* for the 5'-truncated *intR* and *rro*<sup>TS</sup> (future work), respectively. The integrase deletion is needed to prevent *intR* catalysed excision.

30

Because of the extremely low transformation efficiency of *L. lactis* R1 (less than 1 transformant/ $\mu$ g pVE6007) we tried to cure the strain of its natural plasmids. We succeeded in curing two plasmids of approximately 50 kb and 2 kb, by growing *L. lactis* R1 on glucose and incubation at 37 °C. The resulting strain *L. lactis* R131 was shown by UV-induction to still contain R1-t prophage.

35

Sofar we have not succeeded in introducing pBTS1 into *L. lactis* R131. It appears that the prophage is induced after the electroporation. This, together with the presence of MgCl<sub>2</sub> and CaCl<sub>2</sub> in the recovery medium, makes the cells lyse (Table 6).

Therefore, we are currently trying to obtain the pBTS1 integrant by two additional strategies. Firstly, pBTS1 and pVE6007 will be introduced together in *L. lactis* R1K10. pVE6007 encodes a temperature

sensitive RepA protein enabling pBTS1 to replicate. After  
relysogenisation of the resulting double transformant with phage R1-t,  
pBTS1 will integrate into the chromosome when raising the temperature to  
37 °C. The second way to obtain a 'food-grade' inducible lysis system is  
5 to introduce pBTS1 and pVE6007 together in *L. lactis* MG1363. The *attB*-  
region of this strain has been sequenced and appeared to have an homology  
of 99% with the *attB*-region of *L. lactis* R1K10 (Figure 12). *L. lactis*  
MG1363 will be transformed with R1-t DNA ligated at its cos-sites. After  
*intR*-catalysed integration of the R1-t chromosome into the attachment  
10 site of MG1363 (Figure 12), pBTS1 can integrate when raising the  
temperature to 37 °C.

## Figure legends

## Figure 8

5 Cloning scheme for the construction of pBTS1. *EmR*, erythromycin resistance gene; *rro*, R1-t repressor gene; *po*, promoter(p1/p2)/operator region from bacteriophage R1-t; *tec*, topological equivalent of lambda *cro*; *lytP*, R1-t holin gene; *lytR*, R1-t lysin gene; T, transcription terminator of *prtP*; ORI+, origin of replication of the lactococcal  
10 plasmid pWV01; p32, promoter sequence of ORF32 of *L. lactis*; *lacZ*,  $\beta$ -galactosidase gene of *E. coli*; '*intR*', 5'-truncated R1-t integrase gene; *amp*, ampicillin resistance gene. Only relevant restriction enzyme sites are shown.

## 15 Figure 9

Cloning scheme for the construction of pUC18Intd in which the integrase gene of bacteriophage R1-t is 5'-truncated. *intR*, R1-t integrase gene; *lacZ*,  $\beta$ -galactosidase gene of *E. coli*; *amp*, ampicillin resistance gene; '*intR*', 5'-truncated R1-t integrase gene. Only relevant  
20 restriction enzyme sites are shown.

## Figure 10

Effect of mitomycin C on OD600 of *L. lactis* LL108 containing pORI13 (Leenhouts and Venema, 1993) (open square) and pBTS1 (filled  
25 triangle). The cultures were induced with 1  $\mu$ g/ml mitomycin C at time zero (dotted line).

## Figure 11

30 Schematic representation of the food-grade removal of most of the genomic DNA of R1-t prophage in such a way that the inducible regulatory region of the temperate bacteriophage is directly placed upstream of the lysis functions encoded by the prophage. As an example, the integration of pBTS1 in the integrase gene is depicted and the second recombination occurs in the region of the lytic functions.  
35 (A), integrase-region; (B), regulatory-region; (C), region of the lytic functions; *attR*, 'right' phage-host junction; *intR*, R1-t integrase gene; *rro*, R1-t repressor gene; *tec*, topological equivalent of lambda *cro*; PROPAGE, genomic DNA of R1-t prophage; *lytP*, R1-t holin gene; *lytR*, R1-t lysin gene; *attL*, 'left' phage-host junction; *EmR*, erythromycin resis-

tance gene; *lacZ*,  $\beta$ -galactosidase gene of *E. coli*; '*intR*, 5'-truncated R1-t integrase gene. The genes derived from the plasmid pBTS1 are shaded. The primers: attBR, R100, attBL and R40 are listed in table 4.

## 5 Figure 12

Comparison of the *attB* regions of *L. lactis* R1K10 and MG1363. The *attB* site is shaded. Asterisks indicate identical nucleotides.

TABLE 3. Bacterial strains, phages, and plasmid

10	Bacterial strain, phage or plasmid	Relevant characteristic(s)	Source or reference
	Bacterial strains		
15	<i>L. lactis</i> subsp. <i>cremoris</i>		
	LL108	MG1363 (plasmid-free strain) carrying the pWV01 <i>repA</i> gene, and the <i>Cm'</i> gene on the chromosome (high copy)	Leenhouts, unpublished results
20	R1	Original R1-t lysogenic <i>L. lactis</i> subsp. <i>cremoris</i> strain	Lowrie, 1974
	R131	<i>L. lactis</i> R1 cured of two natural plasmids	This study
	R1K10	R1-t indicator strain	lab collection
25	<i>Escherichia coli</i> JM101	<i>supE thi</i> $\Delta$ ( <i>lac-proAB</i> ) [F <i>traD36 proAB<sup>+</sup> lacP<sup>+</sup> lacZ</i> $\Delta$ M15]	Messing, 1979
	Bacteriophage R1-t	type P335, small isometric temperate lactococcal phage, isolated from <i>L. lactis</i> subsp. <i>cremoris</i> R1	Lowrie, 1974
30	Plasmids		
	pIR1PR	Em'	Nauta <i>et al.</i> , 1994 <sup>a,b</sup>
	pORI280	Em'	Leenhouts and Venema, 1993
	pORIR1PR	Em'	This study
	pUC18	Ap'	Yanisch-Perron <i>et al.</i> , 1985
35	pUC18Int	Ap'	This study
	pUC18Intd	Ap'	This study
	pBTS1	Em'	This study
	pVE6007	Cm'	Maguin <i>et al.</i> , 1992
	pORI13	Em'	Leenhouts and Venema, 1993

TABLE 4. Nucleotide sequences of PCR primers

Primer	Sequence	
5		
int1( <i>Sac</i> I)	5'-GCGCGAGCTCCCGCTCAAGTTTGACGACAAGGG-3'	(SEQ id no 9)
int2( <i>Xba</i> I)	5'-GCGCTCTAGAGGATAGATGTGCTTTAGATAATGGC-3'	(SEQ id no 10)
anBL( <i>Xba</i> I)	5'-GCGCTCTAGACAGCTATTCTATCTGTTTCGTAAGGG-3'	(SEQ id no 11)
anBR( <i>Pst</i> I)	5'-GCGCCTGCAGTACCTAAGCACACGAAGGCCTAGG-3'	(SEQ id no 12)
10 R40	5'-CAAATTGGATAGTTAAGG-3'	(SEQ id no 13)
R100	5'-CTCGTGATTACTATTGG-3'	(SEQ id no 14)

\* The restriction enzyme sites are underlined.

15

TABLE 5. Primer-sets to check integration

Strain	Primer-sets <sup>1, 2</sup>	
	anBR/R100	anBL/R40
20 <i>L. lactis</i> R1	2271 bp	-
pBTS1 integrated in:		
<i>intR</i> -region (A)	1687 bp	-
regulatory-region (B)	2271 bp	-
25 lytic-region (C)	2271 bp	2249 bp
After second recomb.	1687 bp	2249 bp

<sup>1</sup> Primers are listed in Table 4.

<sup>2</sup> The sizes of the expected PCR-fragments are given.

30

35

TABLE 6. R1-t induction by electrical pulse

Electrical pulse (kV) <sup>1</sup>	Medium <sup>2</sup>	OD600 of R131 <sup>3</sup>			PFU/ml
		1.5 hrs	4.5 hrs	21.5 hrs	4.5 hrs
0	GSM17	0.323	1.097	2.248	$1.4 \cdot 10^4$
2.5	GSM17	0.228	0.783	2.000	$4.4 \cdot 10^5$
0	GSM17MC	0.242	0.530	1.120	$1.1 \cdot 10^6$
2.5	GSM17MC	0.190	0.189	0.107	$1.9 \cdot 10^7$

<sup>1</sup> Electroporation cuvette (2-mm electrode gap); 25  $\mu$ F/200 $\Omega$

<sup>2</sup> Holo and Ness, 1989.

<sup>3</sup> 40  $\mu$ l competent cells (van der Lelie et al., 1988), cells harvested at OD600 = 0.714

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

## (i) APPLICANT:

(A) NAME: Quest International

(B) STREET: Huizerstraatweg 28

(C) CITY: Naarden

10

(E) COUNTRY: The Netherlands

(F) POSTAL CODE (ZIP): 1411 GP

(G) TELEPHONE: 02159 - 99111

(H) TELEFAX: 02159 - 46067

15

(ii) TITLE OF INVENTION: Process for inhibiting the growth of a culture of lactic acid bacteria, and optionally lysing the bacterial cells, and uses of the resulting lysed culture.

(iii) NUMBER OF SEQUENCES: 8

20

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

25

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EP0)

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

30

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

35

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Lactococcus phage R1-t

(C) INDIVIDUAL ISOLATE: primer LYT1

## 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAAACCCGGG AAGCTTGTCG ACAGCAGTGA TTGGTTCAAC G

41

## 10 (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## 20 (vi) ORIGINAL SOURCE:

(A) ORGANISM: Lactococcus phage R1-t

(C) INDIVIDUAL ISOLATE: primer LYT2

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TTCTAGAAGC TTGCATGCCC CTTCTTTTTT ATTATTGAC

39

## 30 (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs

(B) TYPE: nucleic acid

35 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:



(A) ORGANISM: Lactococcus phage R1-t

(C) INDIVIDUAL ISOLATE: primer LYT3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

5

AAAACCCGGG AAGCTTGTCG ACGATAATAC AGCAAGCCTA GTC

43

(2) INFORMATION FOR SEQ ID NO: 4:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

20

(A) ORGANISM: Lactococcus phage R1-t

(C) INDIVIDUAL ISOLATE: primer LYT4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

25

TTTTTCTAGA AGCTTGCATG CGAAGCGGGG TTAATTTATC C

41

(2) INFORMATION FOR SEQ ID NO: 5:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1200 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Lactococcus phage R1-t

(C) INDIVIDUAL ISOLATE: Fig.3 cds lytP and cds lytR

(ix) FEATURE:

(A) NAME/KEY: CDS

5 (B) LOCATION: 103..328

(ix) FEATURE:

(A) NAME/KEY: CDS

10 (B) LOCATION: 331..1141

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

	TCTACAGGTA CATGGGAAAA TATCGGTTCA GCAGTGATTG GTTCAACGAC AATATATTAT	60
15	TGGAAACGAA CTGCATAAAA AATAAAAAAT AGGAGAAAGA AC ATG AAA ACA TTT	114
	Met Lys Thr Phe	
	1	
	TTT AAA GAT ATG GCA GAA CGT GCC ATT AAA ACA TTT GCA CAA GCA ATG	162
20	Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe Ala Gln Ala Met	
	5 10 15 20	
	ATT GGC GCT TTG GGT GCT GGT GCC ACA GGC TTA ATT GGG GTT GAT TGG	210
25	Ile Gly Ala Leu Gly Ala Gly Ala Thr Gly Leu Ile Gly Val Asp Trp	
	25 30 35	
	CTT CAA GCC TTG AGT ATC GCA GGG TTT GCA ACA GTG GTA TCA ATT CTT	258
	Leu Gln Ala Leu Ser Ile Ala Gly Phe Ala Thr Val Val Ser Ile Leu	
	40 45 50	
30	ACT TCA TTA GCA AGT GGG ATT CCG GGC GAT AAT ACA GCA AGC CTA GTC	306
	Thr Ser Leu Ala Ser Gly Ile Pro Gly Asp Asn Thr Ala Ser Leu Val	
	55 60 65	
35	AAT AAT AAA AAA GAA GGG GAA T AA ATG ACA ATT TAC GAC AAA ACG TTC	354
	Asn Asn Lys Lys Glu Gly Glu Met Thr Ile Tyr Asp Lys Thr Phe	
	70 75 1 5	

	CTA CTC GGC ACA GGT CAA GGT TCG TCA CAA AAG GCG AGT AAT CGA TAT	402
	Leu Leu Gly Thr Gly Gln Gly Ser Ser Gln Lys Ala Ser Asn Arg Tyr	
	10 15 20	
5	ATC GTG ATT CAC GAT ACC GCC AAT GAT AAT AAC CAA GGT GAT AAT AGT	450
	Ile Val Ile His Asp Thr Ala Asn Asp Asn Asn Gln Gly Asp Asn Ser	
	25 30 35 40	
	GCC ACA AAT GAA GCG AGT TAT ATG CAC AAT AAC TGG CAA AAT GCC TAT	498
10	Ala Thr Asn Glu Ala Ser Tyr Met His Asn Asn Trp Gln Asn Ala Tyr	
	45 50 55	
	ACT CAT GCC ATT GCT GGC TGG GAT AAA GTG TAT TTG GTA GGA GAA CCT	546
	Thr His Ala Ile Ala Gly Trp Asp Lys Val Tyr Leu Val Gly Glu Pro	
15	60 65 70	
	GGA TAT GTT GCT TAT GGT GCA GGG AGT CCA GCT AAT GAA CGC TCA CCG	594
	Gly Tyr Val Ala Tyr Gly Ala Gly Ser Pro Ala Asn Glu Arg Ser Pro	
	75 80 85	
20	TTC CAA ATC GAA CTC TCT CAC TAT TCA GAC CCA GCT AAA CAA CGT TCT	642
	Phe Gln Ile Glu Leu Ser His Tyr Ser Asp Pro Ala Lys Gln Arg Ser	
	90 95 100	
25	TCA TAT ATC AAC TAT ATC AAT GCT GTG CGT GAA CAA GCA AAA GTA TTC	690
	Ser Tyr Ile Asn Tyr Ile Asn Ala Val Arg Glu Gln Ala Lys Val Phe	
	105 110 115 120	
	GGT ATC CCT CTT ACT CTT GAT GGA GCA GGT AAT GGT ATC AAA ACT CAT	738
30	Gly Ile Pro Leu Thr Leu Asp Gly Ala Gly Asn Gly Ile Lys Thr His	
	125 130 135	
	AAA TGG GTT TCG GAT AAC CTT TGG GGA GAC CAT CAA GAC CCT TAC TCT	786
	Lys Trp Val Ser Asp Asn Leu Trp Gly Asp His Gln Asp Pro Tyr Ser	
35	140 145 150	
	TAT TTA ACA CGC ATT GGT ATT AGC AAA GAC CAA CTC GCC AAA GAC TTA	834
	Tyr Leu Thr Arg Ile Gly Ile Ser Lys Asp Gln Leu Ala Lys Asp Leu	
	155 160 165	

34

	GCA AAC GGT ATT GGT GGG GCA TCG AAA TCT AAT CAA TCT AAT AAC GAT	882
	Ala Asn Gly Ile Gly Gly Ala Ser Lys Ser Asn Gln Ser Asn Asn Asp	
	170 175 180	
5	GAT TCA ACA CAC GCA ATC AAC TAC ACA CCT AAC ATG GAG GAA AAA GAA	930
	Asp Ser Thr His Ala Ile Asn Tyr Thr Pro Asn Met Glu Glu Lys Glu	
	185 190 195 200	
	ATG ACT TAT CTT ATT TTT GCA AAA GAC ACT AAA CGC TGG TAC ATC ACA	978
10	Met Thr Tyr Leu Ile Phe Ala Lys Asp Thr Lys Arg Trp Tyr Ile Thr	
	205 210 215	
	AAC GGT ATT GAA ATC CGT TAT ATC AAA ACT GGT AGA GTT CTT GGA AAT	1026
	Asn Gly Ile Glu Ile Arg Tyr Ile Lys Thr Gly Arg Val Leu Gly Asn	
15	220 225 230	
	TAT CAA AAT CAA TGG TTG AAA TTC AAA CTT CCT GTG GAT ACT ATG TTC	1074
	Tyr Gln Asn Gln Trp Leu Lys Phe Lys Leu Pro Val Asp Thr Met Phe	
	235 240 245	
20	CAA GCA GAA GTC GAT AAA GAG TTT GGA ACT GGA GCA ACA AAT CCA AAT	1122
	Gln Ala Glu Val Asp Lys Glu Phe Gly Thr Gly Ala Thr Asn Pro Asn	
	250 255 260	
25	CGT GAC ATT TCA AAA GGA T AAATTAACCC CGCTTCGGCG GGTGTTTTTT	1171
	Arg Asp Ile Ser Lys Gly	
	265 270	
	TAAATATAAT TTATTCAAAT AACATTTTT	1200
30		

## (2) INFORMATION FOR SEQ ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 75 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Lys Thr Phe Phe Lys Asp Met Ala Glu Arg Ala Ile Lys Thr Phe  
 1 5 10 15  
 5 Ala Gln Ala Met Ile Gly Ala Leu Gly Ala Gly Ala Thr Gly Leu Ile  
 20 25 30  
 Gly Val Asp Trp Leu Gln Ala Leu Ser Ile Ala Gly Phe Ala Thr Val  
 10 35 40 45  
 Val Ser Ile Leu Thr Ser Leu Ala Ser Gly Ile Pro Gly Asp Asn Thr  
 50 55 60  
 15 Ala Ser Leu Val Asn Asn Lys Lys Glu Gly Glu  
 65 70 75

(2) INFORMATION FOR SEQ ID NO: 7:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 270 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

30 Met Thr Ile Tyr Asp Lys Thr Phe Leu Leu Gly Thr Gly Gln Gly Ser  
 1 5 10 15  
 Ser Gln Lys Ala Ser Asn Arg Tyr Ile Val Ile His Asp Thr Ala Asn  
 20 25 30  
 35 Asp Asn Asn Gln Gly Asp Asn Ser Ala Thr Asn Glu Ala Ser Tyr Met  
 35 40 45

36

His Asn Asn Trp Gln Asn Ala Tyr Thr His Ala Ile Ala Gly Trp Asp  
 50 55 60

Lys Val Tyr Leu Val Gly Glu Pro Gly Tyr Val Ala Tyr Gly Ala Gly  
 5 65 70 75 80

Ser Pro Ala Asn Glu Arg Ser Pro Phe Gln Ile Glu Leu Ser His Tyr  
 85 90 95

10 Ser Asp Pro Ala Lys Gln Arg Ser Ser Tyr Ile Asn Tyr Ile Asn Ala  
 100 105 110

Val Arg Glu Gln Ala Lys Val Phe Gly Ile Pro Leu Thr Leu Asp Gly  
 115 120 125

15 Ala Gly Asn Gly Ile Lys Thr His Lys Trp Val Ser Asp Asn Leu Trp  
 130 135 140

Gly Asp His Gln Asp Pro Tyr Ser Tyr Leu Thr Arg Ile Gly Ile Ser  
 20 145 150 155 160

Lys Asp Gln Leu Ala Lys Asp Leu Ala Asn Gly Ile Gly Gly Ala Ser  
 165 170 175

25 Lys Ser Asn Gln Ser Asn Asn Asp Asp Ser Thr His Ala Ile Asn Tyr  
 180 185 190

Thr Pro Asn Met Glu Glu Lys Glu Met Thr Tyr Leu Ile Phe Ala Lys  
 195 200 205

30 Asp Thr Lys Arg Trp Tyr Ile Thr Asn Gly Ile Glu Ile Arg Tyr Ile  
 210 215 220

Lys Thr Gly Arg Val Leu Gly Asn Tyr Gln Asn Gln Trp Leu Lys Phe  
 35 225 230 235 240

Lys Leu Pro Val Asp Thr Met Phe Gln Ala Glu Val Asp Lys Glu Phe  
 245 250 255

Gly Thr Gly Ala Thr Asn Pro Asn Arg Asp Ile Ser Lys Gly  
 260 265 270

5 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 241 amino acids  
 (B) TYPE: amino acid  
 10 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

15 (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Lactococcus lactis* subsp. *cremoris*  
 (C) INDIVIDUAL ISOLATE: Fig.2 c2 lysin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

20

Leu Phe Pro Tyr Lys Lys Thr Ile Ile Ile Ile Gly Gly Gly Asn Ile  
 1 5 10 15

25

Lys Val Ser Gln Asn Gly Leu Asn Leu Ile Lys Glu Phe Glu Gly Cys  
 20 25 30

Arg Leu Thr Ala Tyr Lys Pro Val Pro Trp Glu Gln Met Tyr Thr Ile  
 35 40 45

30

Gly Trp Gly His Tyr Gly Val Thr Ala Gly Thr Thr Trp Thr Gln Ala  
 50 55 60

35

Gln Ala Asp Ser Gln Leu Glu Ile Asp Ile Asn Asn Lys Tyr Ala Pro  
 65 70 75 80

Met Val Asp Ala Tyr Val Lys Gly Lys Ala Asn Gln Asn Glu Phe Asp  
 85 90 95

38

Ala Leu Val Ser Leu Ala Tyr Asn Cys Gly Asn Val Phe Val Ala Asp  
100 105 110

5 Gly Trp Ala Pro Phe Ser His Ala Tyr Cys Ala Ser Met Ile Pro Lys  
115 120 125

Tyr Arg Asn Ala Gly Gly Gln Val Leu Gln Gly Leu Val Arg Arg Arg  
130 135 140

10 Gln Ala Glu Leu Asn Leu Phe Asn Lys Pro Val Ser Ser Asn Ser Asn  
145 150 155 160

Gln Asn Asn Gln Thr Gly Gly Met Ile Lys Met Tyr Leu Ile Ile Gly  
165 170 175

15 Leu Asp Asn Ser Gly Lys Ala Lys His Trp Tyr Val Ser Asp Gly Val  
180 185 190

Ser Val Arg His Val Arg Thr Ile Arg Met Leu Glu Asn Tyr Gln Asn  
195 200 205

20 Lys Trp Ala Lys Leu Asn Leu Pro Val Asp Thr Met Phe Ile Ala Glu  
210 215 220

25 Ile Glu Ala Glu Phe Gly Arg Lys Ile Asp Met Ala Ser Gly Glu Val  
225 230 235 240

Lys

30



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25

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         *Bacillus* bacteriolytic enzyme in *Escherichia coli*. Mol. Gen.  
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         *Streptococcus pneumoniae* bacteriophage HB-3 amidase reveals  
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## C L A I M S

1. Process for inhibiting the growth of a culture of lactic acid bacteria,  
5 which process comprises the *in situ* production in the cells of the lactic acid bacteria of a holin obtainable from bacteriophages of Gram-positive bacteria, esp. from bacteriophages of lactic acid bacteria, the gene encoding said holin being under control of a first regulatable promoter, said first regulatable promoter not normally being associated with the  
10 holin gene, said holin being capable of exerting a bacteriostatic effect on the cells in which it is produced by means of a system, whereby the cell membrane is perforated, while preferably the natural production of autolysin is not impaired.
2. Process according to claim 1, which additionally comprises the  
15 *in situ* production in the cells of the lactic acid bacteria of a lysin obtainable from grampositive bacteria, preferably lactic acid bacteria or their bacteriophages, the gene encoding said lysin being under control of a second regulatable promoter, said second regulatable promoter not normally being associated with the lysin gene whereby the produced lysin effects lysis of the cells of the lactic acid bacteria.
- 20 3. Process according to claim 2, in which the second regulatable promoter is the same as the first regulatable promoter.
4. Process according to claim 3, in which the gene encoding the holin and the gene encoding the lysin are placed under control of the  
25 same regulatable promoter in one operon.
5. Process according to claim 1 or 2, in which said first or second promoter or both are regulated by food-grade ingredients or parameters.
6. Process according to claim 1 or 2, in which the culture of  
30 lactic acid bacteria is part of a product containing such culture.
7. Process according to claim 6, in which the lactic acid bacteria culture is used for producing a fermented food product obtainable by the fermentative action of the lactic acid bacteria and subsequently the lactic acid bacteria in the fermented food product are lysed.
- 35 8. Process according to claim 7, in which the fermented food product is a cheese product.
9. Process according to claim 8, in which additionally a cheese ripening step is carried out, whereby some of the constituents after leaving the lysed cells will change the composition of the cheese product.

10. Process for combatting spoiling bacteria or pathogenic bacteria, in which a lysed culture obtained by a process as claimed in claim 2 is used as a bactericidal agent.

5 11. Process for improving the shelf life of a consumer product, in which a product obtained by a process as claimed in claim 1 or 2 and containing free holin or free lysin or both is incorporated into said consumer product in such amount that in the resulting consumer product the growth of spoiling bacteria or pathogenic bacteria is inhibited or that their viability is strongly reduced.

10 12. Process according to claim 11, in which the consumer product is selected from the group consisting of edible products, cosmetic products, and products for cleaning fabrics, hard surfaces and human skin.

15 13. Process for modifying a mixture of peptides, which comprises (1) combining a culture of lactic acid bacteria with a mixture of peptides obtained by proteolysis of proteins, the cells of said culture containing both a gene encoding a holin under control of a first regulatable promoter and a gene encoding a lysin under control of a second regulatable promoter, which second and first promoter can be the same, and which second and first promoters are not normally associated with the respective genes and

20 (2) effecting induction of the promoter or promoters for producing both the holin and the lysin in such amounts that the cells of the lactic acid bacteria are lysed and the contents of the cells containing peptidases will modify the composition of the mixture of peptides.

25 14. Process for modifying a mixture of peptides, which comprises treating a mixture of peptides obtained by proteolysis of proteins with a lysed culture obtained by a process according to claim 2.

30 15. Process according to claim 13 or 14, in which the proteins comprise milk proteins or vegetable proteins, or both.

16. Process according to any of the preceding claims, wherein the holin is encoded by a nucleic acid sequence according to any of claims 18-20 and/or is expressed from a recombinant vector according to any of claims 21-24 and/or is expressed by a recombinant cell according to any  
35 of claims 25-27.

17. Process according to any of claims 2-16, wherein the lysin has the amino acid sequence of sequence id no 7 or is a functional equivalent thereof.

18. A nucleic acid sequence encoding a holin derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a *L. lactis* or a bacteriophage derivable from such a grampositive bacterium.
- 5 19. A nucleic acid sequence according to claim 18 encoding the amino acid sequence of sequence id no 6 or or a functional equivalent thereof such as the nucleic acid sequence of nucleotides 103-328 of sequence id no 5.
- 10 20. A nucleic acid sequence according to claim 18 or 19, further being operatively linked to a first regulatable promoter, said first regulatable promoter not normally being associated with the holin encoding sequence.
- 15 21. A recombinant vector comprising a nucleic acid sequence according to any of claims 18-20, said vector preferably further being foodgrade.
- 20 22. A recombinant vector according to claim 21 further comprising a nucleic acid sequence encoding a lysin, both the holin and the lysin being derivable from a grampositive bacterium such as a lactic acid bacterium, in particular a *L. lactis* or a bacteriophage derivable from such a grampositive bacterium.
- 25 23. A recombinant vector according to claim 21 or 22 further comprising a the natural attachment/integration system of a bacteriophage for example said system comprising the bacteriophage attachment site and an integrase gene located such that integration of the holin and optionally lysin gene will occur, said system preferably being derived from a bacteriophage that is derivable from a food grade host cell, preferably a lactic acid bacterium.
- 30 24. A recombinant vector according to any of the claims 21-23, wherein the nucleic acid sequence encoding the holin and the nucleic acid sequence encoding the lysin are operatively linked to a foodgrade inducible promoter that can be induced via a food grade mechanism, for example by being a thermosensitive complex inducible promoter.
- 35 25. A recombinant host cell comprising a nucleic acid sequence according to any of claims 18-20 in a setting other than in its native bacteriophage and/or a recombinant vector according to any of claims 21-24.
26. A recombinant host cell according to claim 25 further comprising a nucleic acid sequence encoding a lysin, said lysin preferably being derivable from a grampositive bacterium such as a lactic



acid bacterium, in particular a *L. lactis* or a bacteriophage derivable from such a grampositive bacterium, said nucleic acid sequence encoding a lysin preferably being in a setting other than in its native bacteriophage or bacterium.

- 5 27. A recombinant host cell according to claim 25 or 26 being a food grade host cell, preferably a lactic acid bacterium, most preferably the host cell is of the same type from which the holin and/or lysin encoding nucleic acid sequences are derived

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Fig. 1A

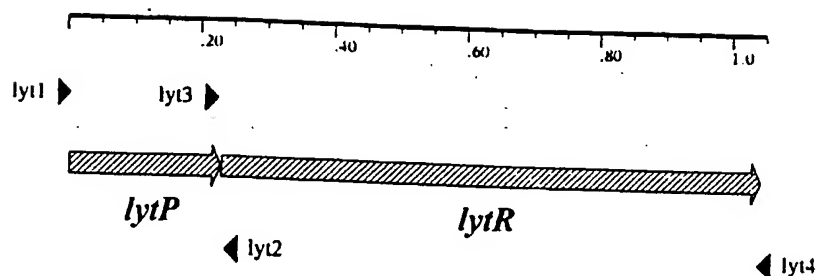
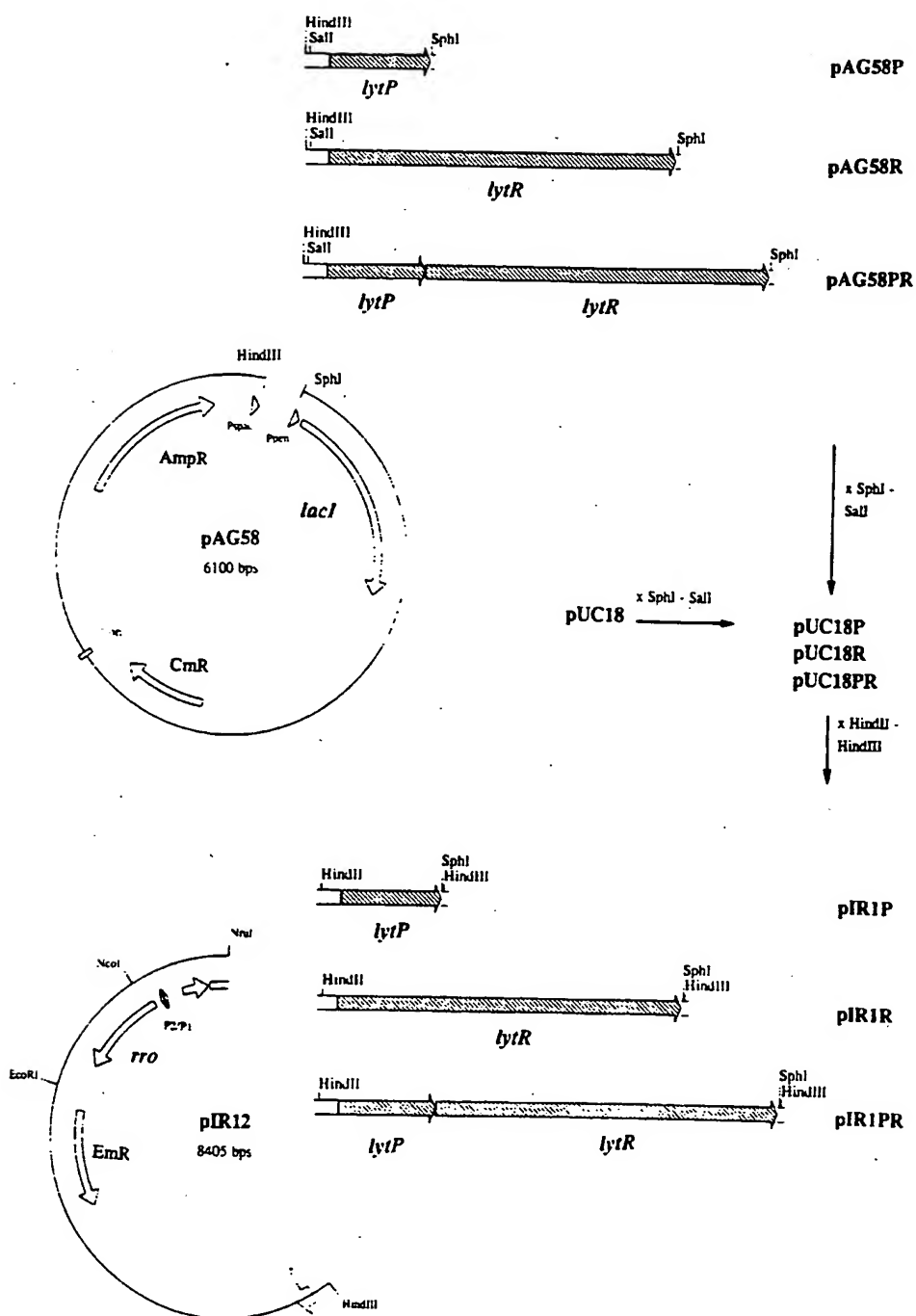


Fig. 2

ORF 23	MTIYDKTFLLGTGQGSSQKASNRYIVIHDTANDNNQGDNSATNEASYMHN	50
c2	LFPYKKTIII-IGGGNIKVSQGNLNIKEFE-----GCRLTAYKPV	40
	* ** .. * * . * . * . . *	
ORF 23	NWQNAVTHAIAGWDKVVLVGEPTYVAYGAGSPANERSPFQIELSHYSDPA	100
c2	PWEQMYT---IGWGHYGVTAGTTW-----TQAQADSQLEIDINNKYAPM	81
	* . ** .. ** . . . . * . * * * . *	
ORF 23	KORSSYINYINAVREQAKVFGIPLTLD-GAGNGIKTHKWVS-DNLWGDHQ	148
c2	VD-----AYVKG-KANQNEFDALVSLAYNCGNVFVADGWAPFSHAYCASM	125
	* . . . . * . . * . ** . *	
ORF 23	DPYSYLTRIGISKDQLAKDLANGIGGASKSNQSNDDSTHAINYTPNMEE	198
c2	IP-KYRNAGGQVLQGLVRRRQAEINLFNKPVSNSNQNNOT-----GG	167
	* * * * * . * . . * . * . . *	
ORF 23	KEMTYLIFAKD----TKRWYITNGIEIRYIKTGRVLGNYQNQWLKFKLPV	244
c2	MIKMYLIIGLDNSGKAKHWYVSDGVSVRHVRTIRMLENYQNKWAKLNLPV	217
	*** * . * * * . * . . * * * * * * * * *	
ORF 23	DTMFQAEVDKEFGTGATNPNRDISKG	270
c2	DTMFIAEIEAEFGRKIDMASGEVK--	241
	***** * * . . *	

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Fig. 1B

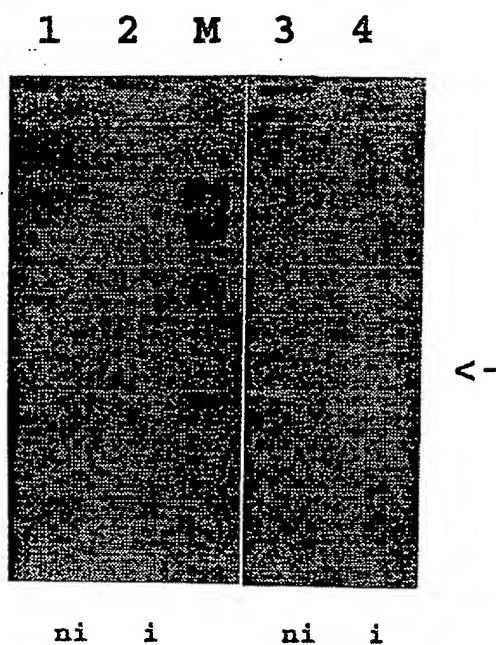


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Fig. 3

TCTACAGGTACATGGGAAAATATCGGTTCAAGCAGTGATTGGTTCAACGACAATATATTAT  
 TGGAAACGAACTGCATAAAAAATAAAAAATAGGAGAAAGAACATGAAAACATTTTTTAA  
 RBS ----> *lytP*  
 M K T F F K  
 GATATGGCAGAACGTGCCATTAAACATTTGCACAAGCAATGATTGGCGCTTTGGGTGCT  
 D M A E R A I K T F A Q A M I G A L G A  
 GGTGCCACAGGCTTAATTGGGGTTGATTGGCTTCAAGCCTTGAGTATCGCAGGGTTTGCA  
 G A T G L I G V D W L Q A L S I A G F A  
 ACAGTGGTATCAATTCTTACTTCATTAGCAAGTGGGATTCCGGGCGATAATACAGCAAGC  
 T V V S I L T S L S S G I P G D N T A S  
 CTAGTCAATAATAAAAAAGAGGGGAATAAATGACAATTTACGACAAAACGTTCTACTC  
 RBS ----> *lytR*  
 L V N N K K E G E \* M T I Y D K T F L L  
 GGCACAGGTCAAGGTTCTGTCACAAAAGGCGAGTAATCGATATATCGTGATTACGATACC  
 G T G Q G S S Q K A S N R Y I V I H D T  
 GCCAATGATAATAACCAAGGTGATAATAGTGCCACAAATGAAGCGAGTTATATGCACAAT  
 A N D N N Q G D N S A T N E A S Y M H N  
 AACTGGCAAAATGCCTATACTCATGCCATTGCTGGCTGGGATAAAGTGATTTGGTAGGA  
 N W Q N A Y T H A I A G W D K V Y L V G  
 GAACCTGGATATGTTGCTTATGGTGCAGGGAGTCCAGCTAATGAACGCTCACC GTTCCAA  
 E P G Y V A Y G A G S P A N E R S P F Q  
 ATCGAACTCTCTCACTATTAGACCCAGCTAAACAACGTTCTTCATATATCAACTATATC  
 I E L S H Y S D P A K Q R S S Y I N Y I  
 AATGCTGTGCGTGAACAAGCAAAAGTATTCGATATCCCTCTTACTCTTGATGGAGCAGGT  
 N A V R E Q A K V F G I P L T L D G A G  
 AATGGTATCAAACTCATAAATGGGTTTCGGATAACCTTTGGGGAGACCATCAAGACCTT  
 N G I K T H K W V S D N L W G D H Q D P  
 TACTCTTATTTAACACGCATTGGTATTAGCAAAGACCAACTCGCCAAAGACTTAGCAAAC  
 Y S Y L T R I G I S K D Q L A K D L A N  
 GGTATTGGTGGGGCATCGAAATCTAATCAATCTAATAACGATGATTCAACACACGCAATC  
 G I G G A S K S N Q S N N D D S T H A I  
 AACTACACACCTAACATGGAGGAAAAAGAAATGACTTATCTTATTTTGCAAAAGACACT  
 N Y T P N M E E K E M T Y L I F A K D T  
 AAACGCTGGTACATCACAACGGTATTGAAATCCGTTATATCAAACTGGTAGAGTTCTT  
 K R W Y I T N G I E I R Y I K T G R V L  
 GGAAATTATCAAAATCAATGGTTGAAATTCAAACCTCCTGTGGATACTATGTTCCAAGCA  
 G N Y Q N Q W L K F K L P V D T M F Q A  
 GAAGTCGATAAAGAGTTTGGAACTGGAGCAACAAATCCAAATCGTGACATTTCAAAAGGA  
 E V D K E F G T G A T N P N R D I S K G  
 TAAATTAACCCGCTTCGGCGGGTGTTTTTTTAAATATAATTTATTCAAATAACATTTTT  
 \* -----> <-----

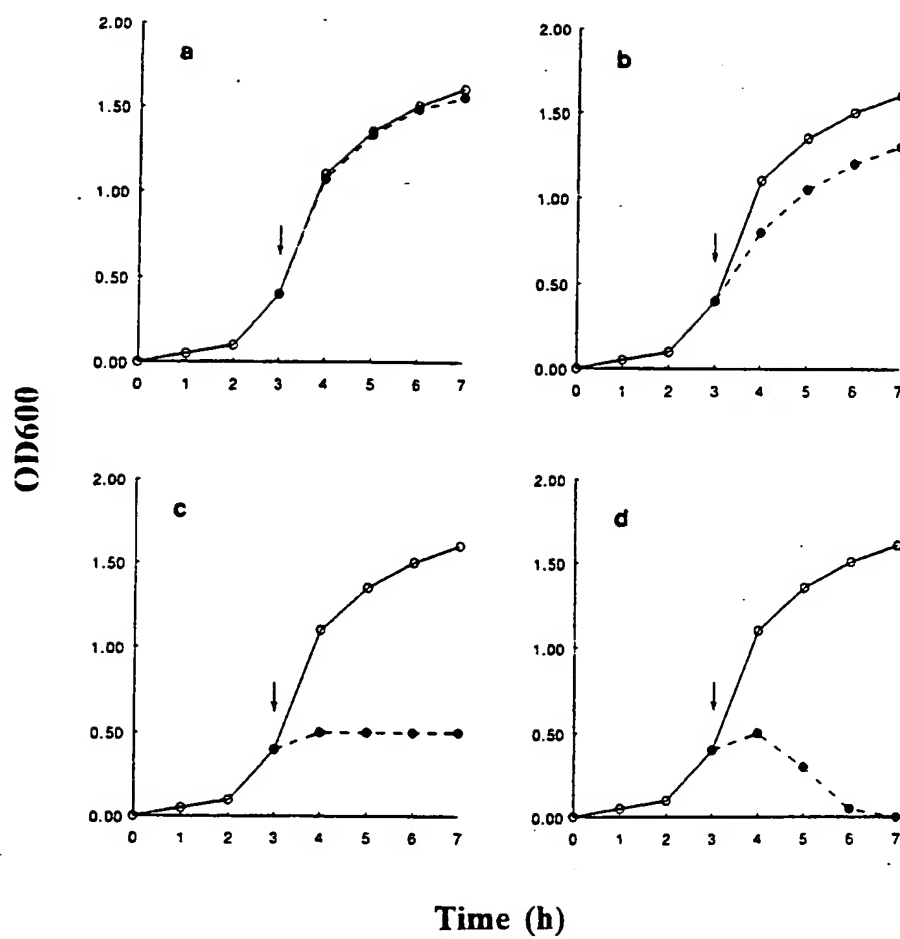
Figure 4



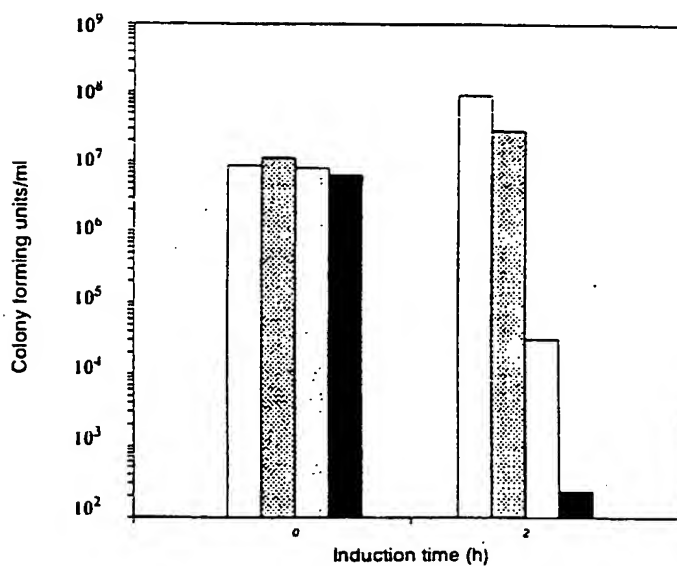


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Fig. 6A

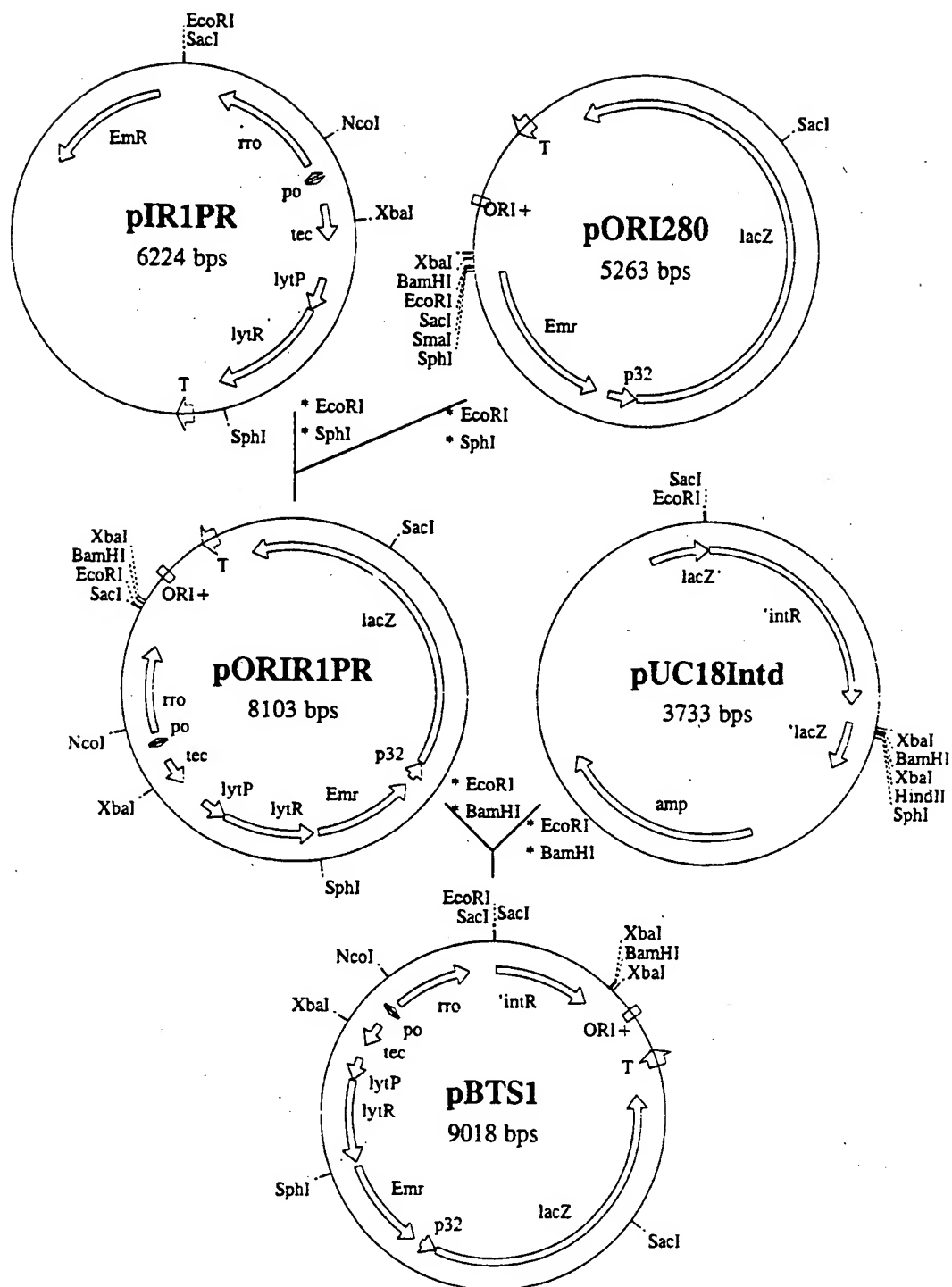


B



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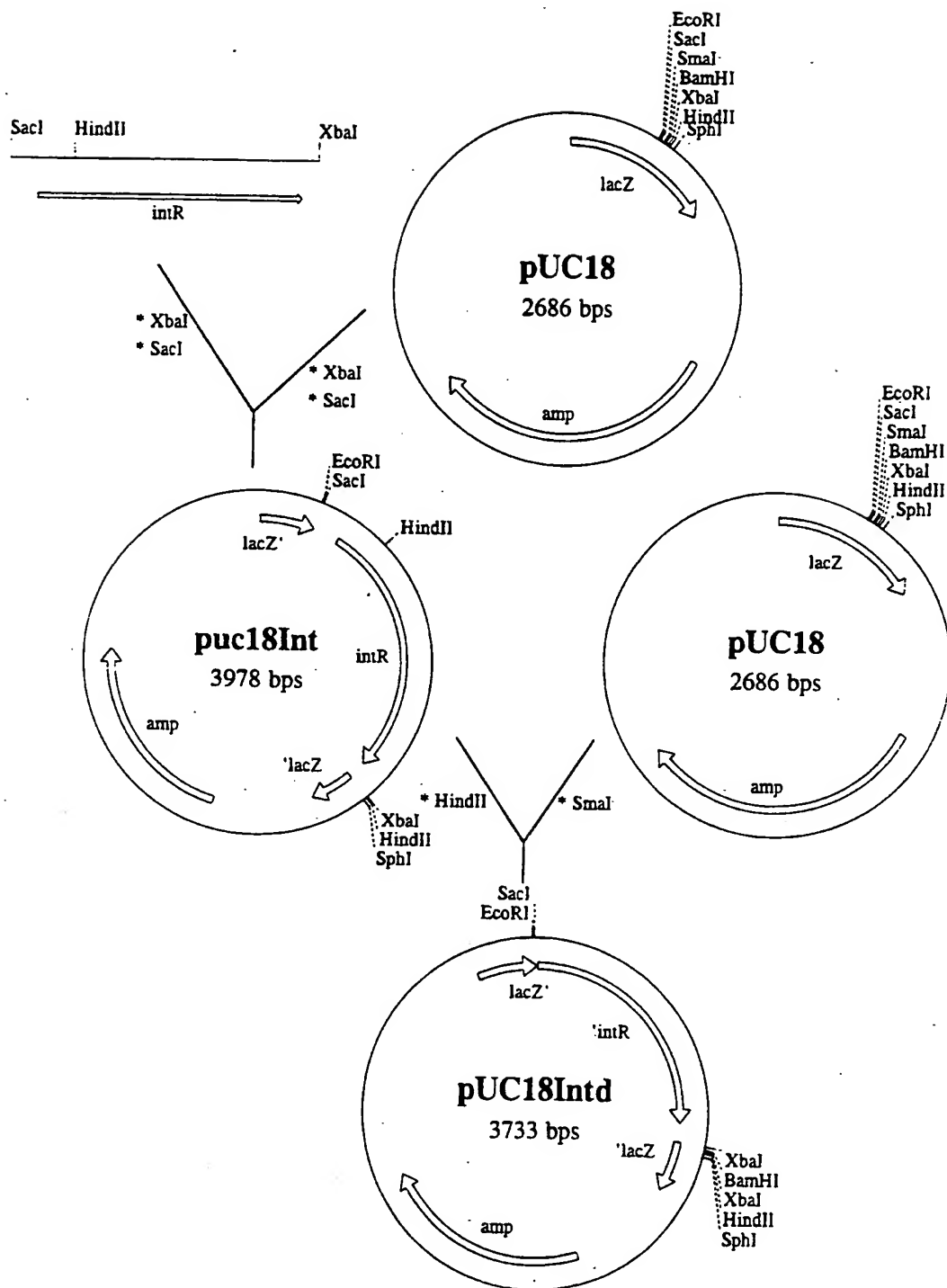
Fig. 8





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Fig. 9



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Fig.10

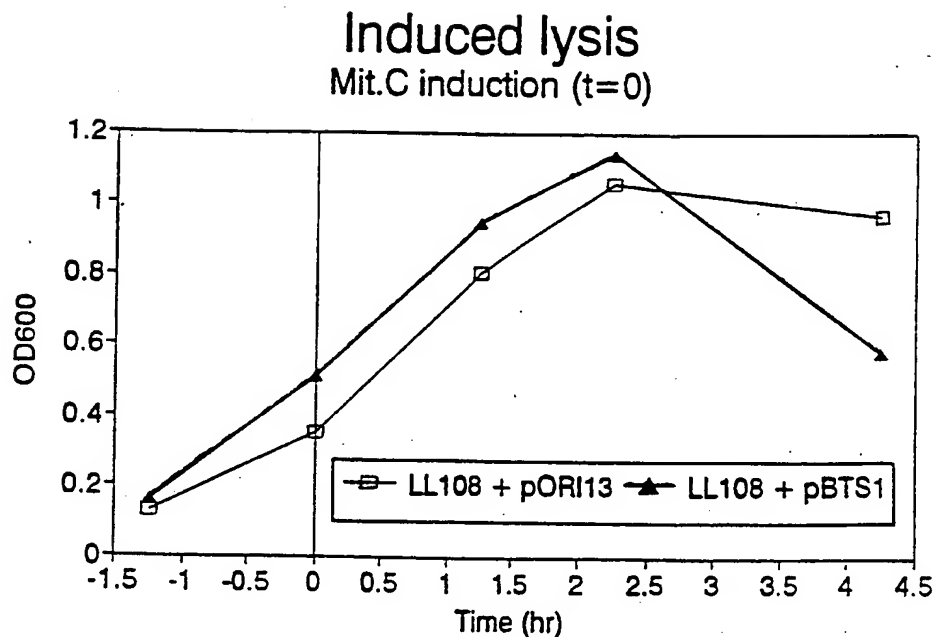


Fig.12

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RIK10  ACAGCTATTCTATCTGTTTCGTAAGGAAAACGCTCAGGAAGTGGTTGATAC  50
MG1363 ACAGCTATTCTATCTGTTTCGTAAGGAAAACGCTCAGGAAGTGGTTGATAC  50
*****

RIK10  CCTTGTTAAAGCAGGAATCAAAGGCTTTCTTAACTTTGCACCTATTTCGCT  100
MG1363 CCTTGTTAAAGCAGGAATCAAAGGCTTTCTTAACTTTGCACCTATTTCGCT  100
*****

RIK10  TGAAAGTCCCTTCAGATGTTGTTGTTCATCTATTGATTTAACTAAAGAA  150
MG1363 TGAAAGTCCCTTCAGATGTTGTTGTCCAATCTATTGATTTAACTAAAGAA  150
*****

RIK10  TTGCAAACCTTGTTATTCTTCATGGGAGCTCAAGAAGAATAAAAGACAAG  200
MG1363 TTGCAAACCTTGTTATTCTTCATGGGAGCTCAAGAAGAATAAAAGACAAG  200
*****

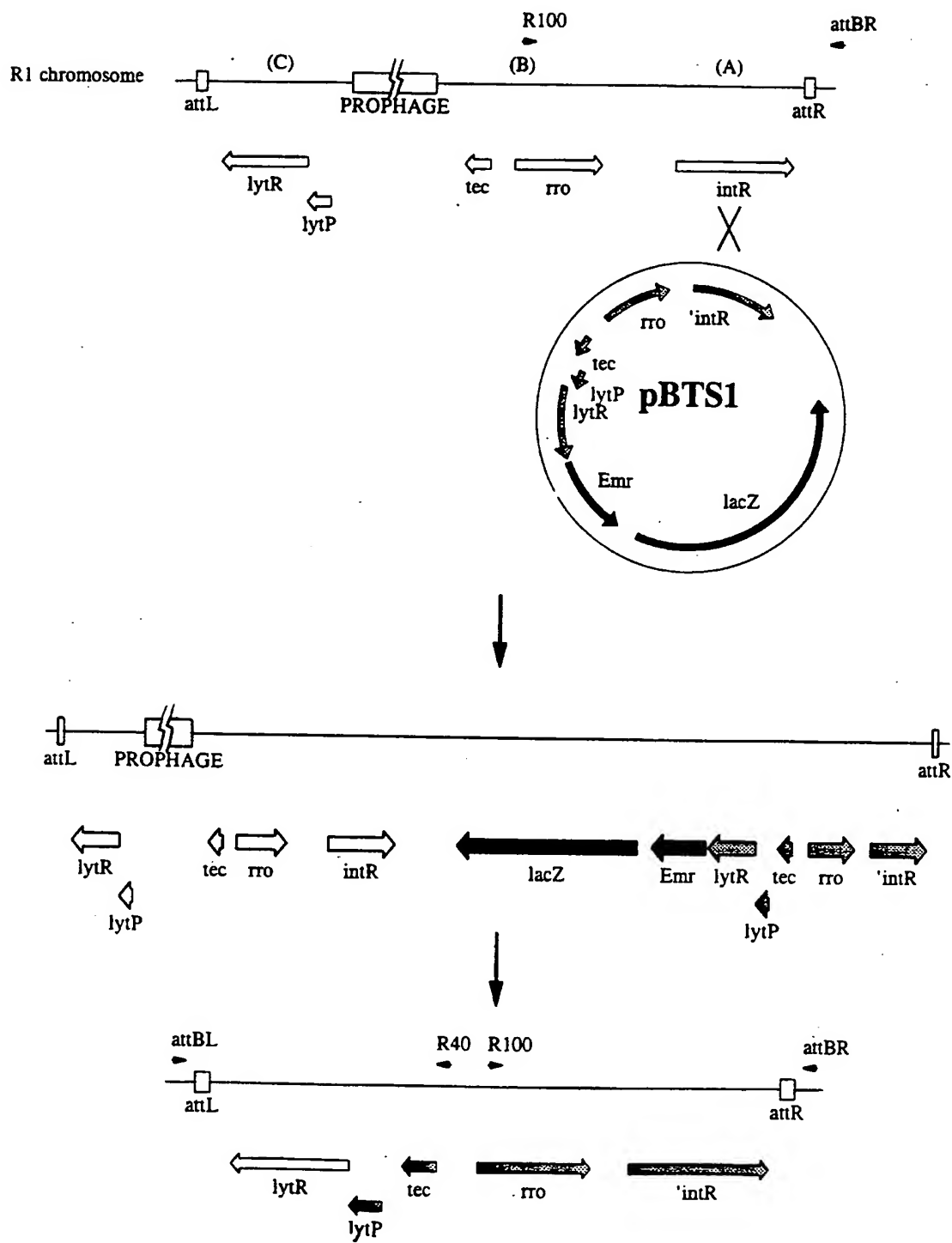
RIK10  CAAAATTTTATCAAAACTATTAATGATACCTAGGCCTTCGTGTGCTTAGG  250
MG1363 CAAAATTTTATCAAAACTATTAATGATACCTAGGCCTTCGTGTGCTTAGG  250
*****

RIK10  TA 252
MG1363 TA 252
**

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Fig.11



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NL 95/00171

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/74 C07K14/315 C12N9/14 A23C19/032		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N A23C C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY, vol. 175, no. 4, 1993 pages 1038-1042, M. STEINER ET AL. 'The missing link in phage lysis of gram-positive bacteria : gene 14 of Bacillus subtilis phage sigma 29 encodes the functional homolog of lambda S protein' cited in the application	18,21
Y	see the whole document  <div style="text-align: center;">--- -/--</div>	1-17, 21-27
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
* Special categories of cited documents :		
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Date of the actual completion of the international search  <div style="text-align: center;">27 September 1995</div>		Date of mailing of the international search report  <div style="text-align: center;">17. 10. 95</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer  <div style="text-align: center;">Gurdjian, D</div>

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PCT/NL 95/00171

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF GENERAL MICROBIOLOGY, vol. 138, COLCHESTER GB, page 1949 LAZAREVIC, VLADIMIR ET AL 'Sequencing and analysis of the Bacillus subtilis lytRABC divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier' see the whole document ---	1-17, 21-27
Y	BIOTECHNOLOGY, vol. 10, 1992 NEW YORK US, page 196 SHEARMAN C.A. ET AL. 'Autolytic lactococcus lactis ...' cited in the application see the whole document ---	1-17, 21-27
A	WO,A,90 00599 (AGRICULTURAL & FOOD RESEARCH COUNCIL) 25 January 1990 cited in the application see abstract; claims 1-17 ---	7-11
Y	EP,A,0 510 907 (AGRICULTURAL AND FOOD RESEARCH COUNCIL) 28 October 1992 cited in the application see claims 1-14 ---	10-12, 21-25
A	GENE., vol. 118, AMSTERDAM NL, page 115 PLATTEEUW C. ET AL. cited in the application see the whole document ---	1-27
P,X	MICROBIOLOGY, vol. 140, 1994 pages 3061-3069, C.SCHOULER ET AL. 'Sequence and organization of the Lactococcal prolate-headed bIL67 phage genome' see the whole document ---	18,21
P,X	CANADIAN J.MICROBIOL., vol. 40,no. 8, 1994 pages 658-665, N.K.BIRKELAND 'Cloning, molecular characterization, and expression of the genes encoding the lytic functions of lactococcal bacteriophage sigmaLC3 : a dual lysis system of modular design' see the whole document ---	18,21
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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 95/00171

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60,no. 6, June 1994 pages 18785-1883, E.K.ARENDT ET AL. 'Molecular characterization of lactococcal bacteriophage Tuc 2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins' see the whole document -----	18,21

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 95/00171

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9000599	25-01-90	AU-B- 623166	07-05-92
		AU-B- 4037789	05-02-90
		EP-A- 0425572	08-05-91
		GB-A- 2243611	06-11-91
		US-A- 5360617	01-11-94
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EP-A-0510907	28-10-92	GB-A- 2255561	11-11-92
		AU-B- 650737	30-06-94
		AU-B- 1502392	22-10-92
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